Phenotypic changes in invasive species: roles of rapid adaptation and historical factors

Ariane Le Gros

UNIVERSITE PARIS.DIDEROT (Paris 7)
SORBONNE PARIS CITE

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Ariane LE GROS

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Phenotypic changes in invasive species: roles of rapid adaptation and historical factors

Changements phénotypiques chez les espèces invasives : rôles de l'adaptation rapide et des facteurs historiques

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Thèse dirigée par Philippe CLERGEAU et Sarah SAMADI

JURY

M. Pierre-André CROCHET	Chargé de Recherche, CEFE – Montpellier	Rapporteur
Mme Sabrina RENAUD	Directrice de Recherche, Université Lyon 1	Rapportrice
Mme Emmanuelle BAUDRY	Maître de Conférences, Université Paris Sud	Examinatrice
M. Vincent BELS	Professeur, MNHN – Paris	Examinateur
M. Philippe CLERGEAU	Professeur, MNHN – Paris	Directeur de thèse
Mme Sarah SAMADI	Professeur, MNHN – Paris	Directrice de thèse

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I.1. Context

I.1.1. Why study rapid evolution?

I.1.1.1. Definition of rapid evolution and implications for Ecology

Although the existence of rapid evolutionary processes has been known for a long time for example in agriculture, rapid evolution is a fairly new concept in Ecology. According to a search done on Web of Science in the domains of Ecology, Evolutionary Biology and Biodiversity Conservation, the terms "rapid evolution" first appeared sporadically in the literature during the 1970s. The number of articles using these terms started to be more frequent in the late 1990s and was multiplied by ten between 2000 and 2013 (figure 1). Rapid evolution, also called contemporary evolution, is defined slightly differently according to authors. For example Carroll *et al.* (2007) defined it as "an evolutionary change, occurring over tens of generations or fewer", whereas Stockwell *et al.* (2003) talk about "an heritable trait evolution observed in contemporary time (*i.e.* less than a few hundred generations)" and Kopp & Matuszewski (2014) present it as "an evolutionary change observed in present-day populations". There is therefore no consensus on the length of time in which an evolutionary change is considered as rapid.

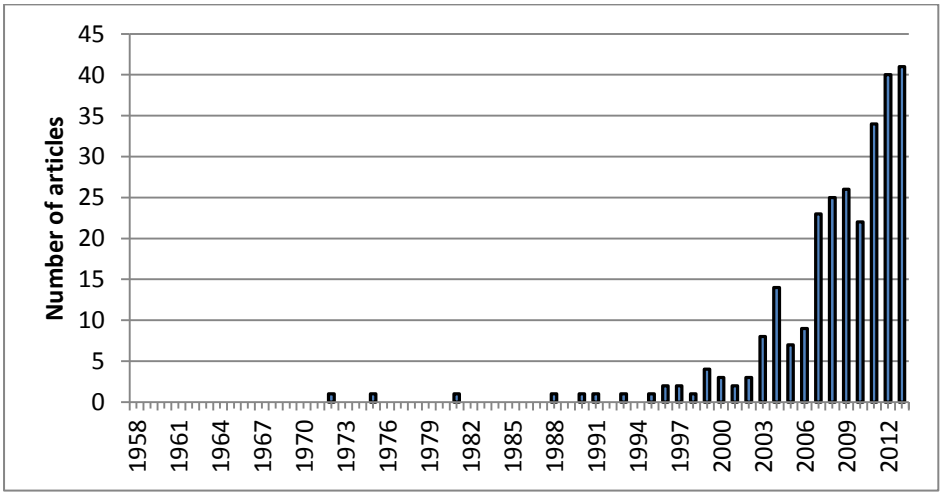

Fig. 1: Evolution of the number of articles published per year and containing the terms "rapid evolution" in the fields of Ecology, Evolutionary Biology and Biodiversity Conservation. These figures were obtained with a search on Web of Science and only research articles were kept. Articles on phylogeny, evolution of genes and proteins, or on unicellular organisms were excluded as the time scale considered for rapid evolution was different than ours.

Despite this lack of consensus, all definitions agree on the fact that rapid evolution can be observed on ecological time-scales and thus interact with ecological processes and affect population dynamics (Lambrinos 2004). This founding raised awareness about the need of including evolution in ecological studies. Theoretical population dynamics studies that included evolutionary process confirmed that rapid evolution can have a strong impact on the outputs of population dynamics models (Mougi & Nishimura 2008; Mougi 2012; Cortez & Weitz 2014). For example Cortez and Weitz (2014) incorporated rapid evolution to classical prey-predator models. They showed that the rate of evolution affects the dynamics obtained with classical models (figure 2).

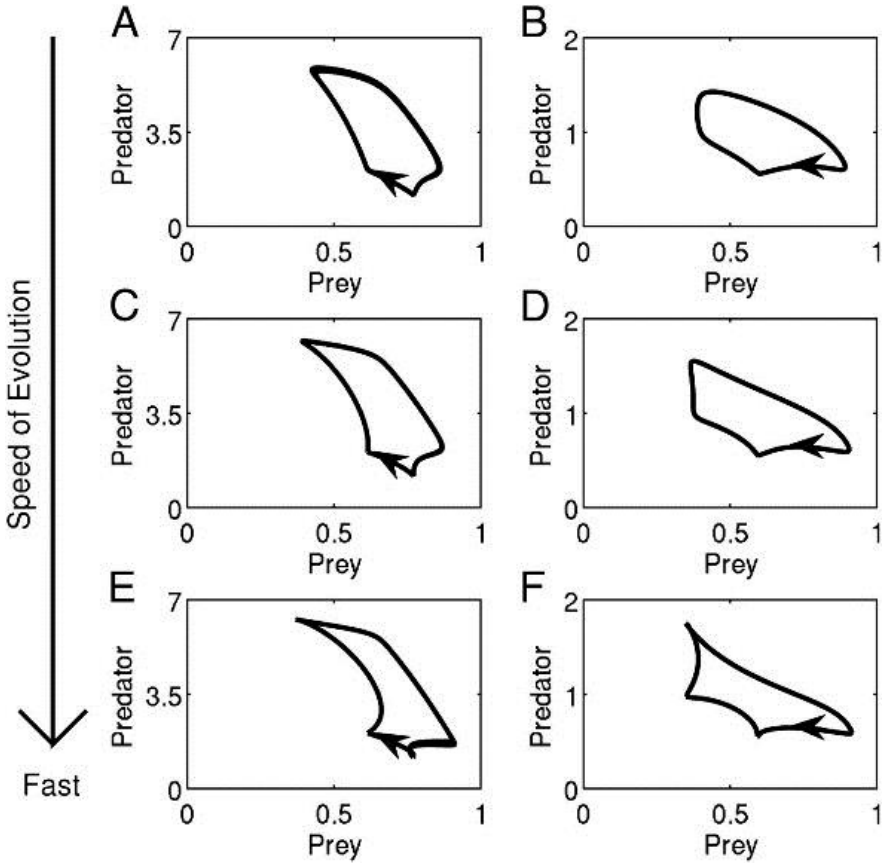

Fig. 2: Changes in predator prey cycles according to the speed of evolution in two examples of clockwise predator–prey cycles. The speed of evolution is (*A* and *B*) as fast, (*C* and *D*) two times as fast, and (*E* and *F*) five times as fast as the ecological dynamics of the system. In these models, the prey exhibit logistic growth in the absence of predation and predation rates follow a Type II functional response. In *A*, *C*, and *E*, the predators have a linear death rate and in *B*, *D*, and *F* the predators have a nonlinear death rate. From Cortez & Weitz (2014)**.**

Considering the potential impact of rapid evolution on ecological processes, some authors underlined the importance of taking evolutionary processes into accounts in applied fields of ecology such as biodiversity management (Hendry *et al.* 2011; Lankau *et al.* 2011). For example in order to preserve a fragmented endangered population, it is essential to favor connectivity and gene flow between patches to favor genetic diversity and evolutionary potential (Lankau *et al.* 2011). If other conservation actions, such as protecting the current habitat of the species, are taken without considering evolutionary aspects, the species might be protected on the short term but is bound to get extinct in the long term. Indeed, it will lack the evolutionary potential necessary to adapt to future changes. In biological control, there are many cases in which evolution was not taken into account. This lead to results opposite to those expected. Antibiotic, insecticide and herbicide resistance are famous examples of cases in which the potential of organisms to evolve a resistance when confronted to a strong selective pressures was not considered (Hemingway & Ranson 2000; Palumbi 2001). It then took only a few years for bacteria, insects and plants to evolve resistance to antibiotics, insecticide and herbicide (figure 3) causing issues in human-health and economy.

EVOLUTION OF RESISTANCE TO ANTIBIOTICS AND HERBICIDES

Antibiotic or herbicide	Year deployed	Resistance observed
	Antibiotics	
Sulfonamides	1930s	1940s
Penicillin	1943	1946
Streptomycin	1943	1959
Chloramphenicol	1947	1959
Tetracycline	1948	1953
Erythromycin	1952	1988
Vancomycin	1956	1988
Methicillin	1960	1961
Ampicillin	1961	1973
Cephalosporins	1960s	late 1960s
	Herbicides	
2,4-D	1945	1954
Dalapon	1953	1962
Atrazine	1958	1968
Picloram	1963	1988
Trifluralin	1963	1988
Triallate	1964	1987
Diclofop	1980	1987

Fig. 3: Year of deployment and year of first observed resistance for representative antibiotics and herbicides, showing the rapidity of resistance evolution. Adapted from (Palumbi 2001).

I.1.1.2. Global changes and biodiversity loss

Since the end of the 1990s, there has been a growing interest in the scientific community for rapid evolution (figure 1). Among the reasons that made rapid evolution, and more specifically rapid adaptation, a popular research topic, a major one is the general concern for biodiversity loss and the hope that adaptation might help at least some species to avoid extinction (25% of the articles on rapid evolution we found). Indeed, human activities are causing fast changes in the environmental conditions on Earth. These changes were classified in five categories: habitat disruption (modification, fragmentation, and destruction), climate change, pollution, over-exploitation of resources and introduction of invasive species (Millennium Ecosystem Assesement (MEA) 2005). Below are a few figures illustrating the evolution of these global changes. Habitat disruption: over the world, about 2.7 million hectares of forest were lost between 1990 and 2000 and twice more (6.3 million hectares) were lost between 2000 and 2005 (Food and Agriculture Organization (FAO) 2010). Climate change: during the last 100 years, the global mean surface temperature has increased by about 0.6 Celsius and is projected to increase from 1990 to 2100 by 1.4–5.8 Celsius. The occurrence of extreme climatic events such as heat waves, drought and floods is also projected to increase in this period (MEA 2005). Pollution: nitrogen application has increased fivefold since 1960, and up to 50% of the nitrogen fertilizer applied is lost to the environment (MEA 2005). Over-exploitation of resources: the FAO estimates that about half of the wild marine fish stocks for which information is available are fully exploited. Introduction of invasive species: in France, there were 18 invasive species of vertebrates during the period 1800-1914, this figure raised to 21 during the period 1914-1945 and to 79 to during the period 1945-2004 (Pascal *et al.* 2006).

These examples, show that human mediated global changes are going faster and faster. They have resulted in the most massive extinction since the apparition of life, and this pattern is predicted to intensify in the future (MEA 2005; figure 4). Conservation biologists are thus wondering whether species threatened by global changes will be able to adapt fast enough to avoid extinction. This concept, called evolutionary rescue (Bell & Gonzalez 2009) is however still mainly theoretical.

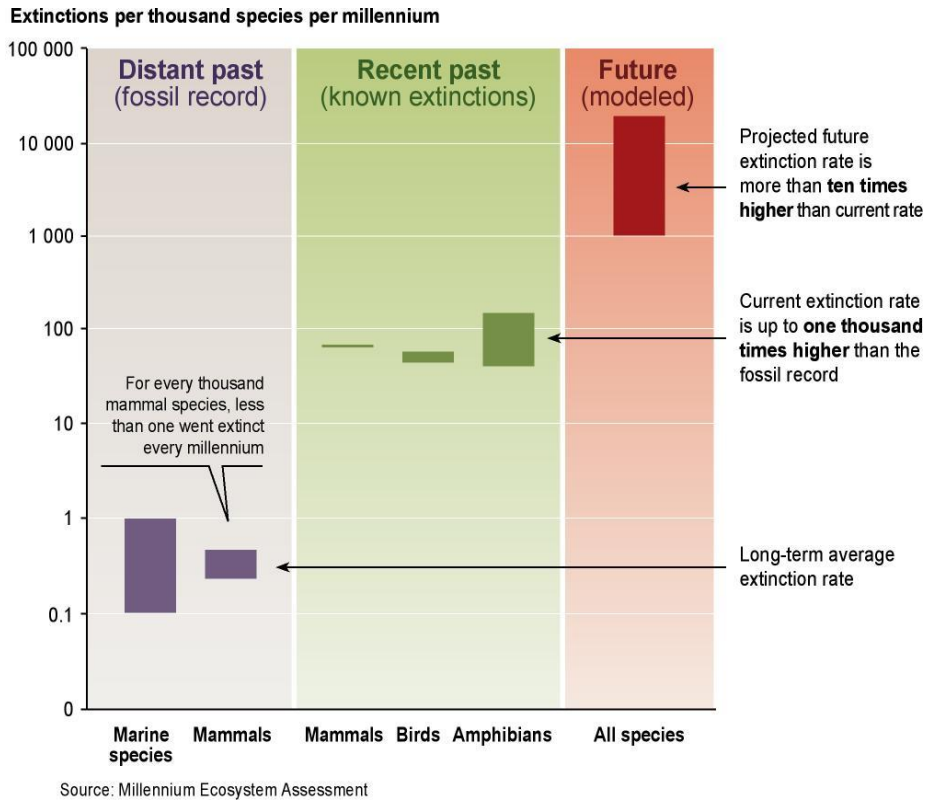

Fig. 4: Evolution with time of rates of extinction in thousand species per millennium (MEA 2005).

I.1.1.3. Can rapid adaptation rescue species from extinction?

Response of species, communities and ecosystems to climate change is a research topic that has received a lot of attention recently. At the species level, the most often observed responses to climate change are populations decline, changes in phenology (mainly breeding timing and migration timing for migratory species), habitat shift towards the poles or higher elevations, and contraction of habitat ranges for species that cannot move farther (reviewed in McCarty (2001)). For example, the abundance of Sooty Shearwaters (*Puffinus griseus*) in California declined by 90% in 7 years in association with a rapid warming of the California current (Veit *et al.* 1997). Seven studies on a total of 29 bird species showed an advance in their breeding date going from 3 to 30 days in a period of 24 to 35 years (McCarty 2001). Devictor *et al.* (2008) showed on 105 bird species in Europe, a 91 Km northward shift of bird communities in the last 17 years. Similarly, Parmesan *et al.* (1999) showed a 35 to 240 Km northward shift over the past century in 63% of the 35 butterfly species they studied (figure 5A). Upward changes of 50 to 1000 m in the range limits of 16 small mammal species of the Yosemite National Park, California, USA were pointed out by Moritz *et al.* (2008). They also showed a frequent range contraction for high-elevation species (figure 5B).

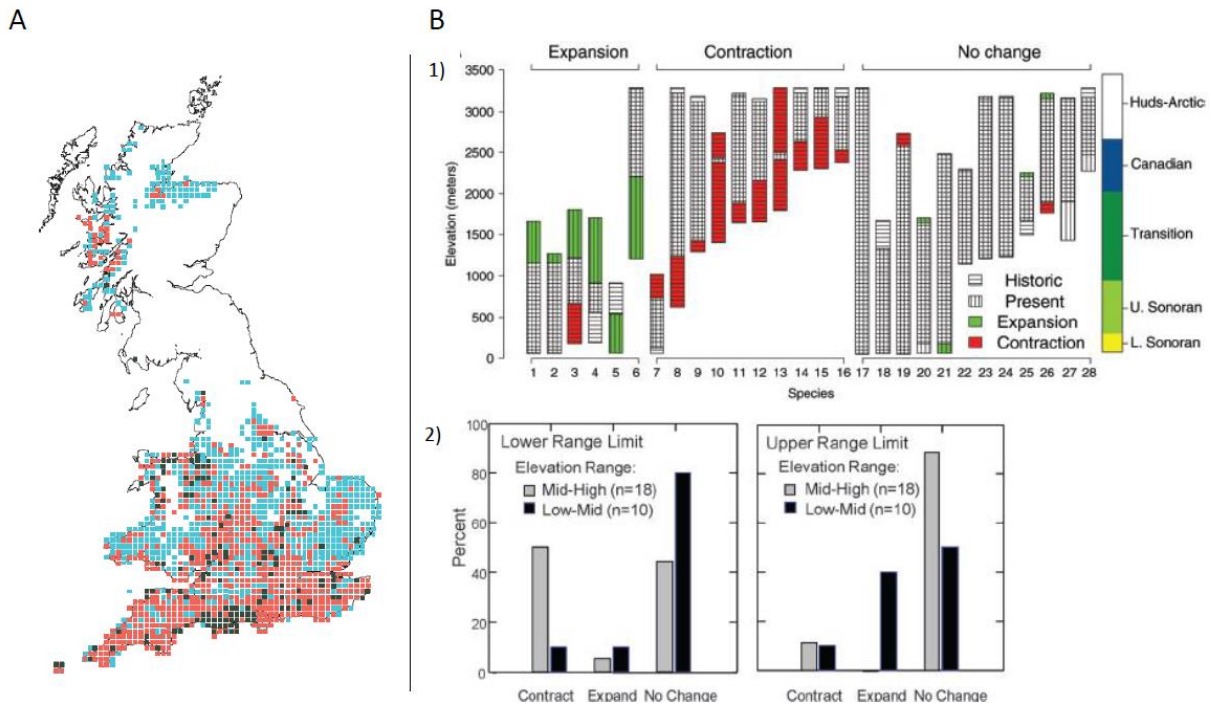

Fig. 5: Examples of latitudinal and altitudinal range shits due to climate change. A) Twentieth-century changes in the range of the butterfly *Pararge aegeria* in Great Britain. A coloured grid cell indicates more than one population in 1915–1939 (black), 1940–1969 (red) or 1970–1997 (blue). From (Parmesan *et al.* 1999). B) (1) Summary of elevational range changes across all species in relation to life zones. Significant ($P < 0.05$) shifts are colored green for range expansion and red for contraction. Species were classified as "No Change" if range shifts were biologically trivial (<10% of previous elevation range) or of small magnitude (<100 m). (2) Comparison of changes in elevation-range limits for species that formerly had low- to mid-elevation versus mid- to high-elevation ranges across the transect. From Moritz *et al.* (2008).

As the possibility of moving towards the poles or higher elevations is not infinite, shifts in habitat range can only be a temporary help. Moreover there are some suspicions about the capacity of species to shift their range sufficiently to face climate change. Devictor *et al.* (2008) showed that bird species are not moving fast enough to follow their historical climate envelop and are lagging 282 Km behind it despite a 91 Km northward shift in 17 years. Thus adaptation to new climatic conditions might be necessary to survive to climate change. Changes in phenology show that some species can respond to climate change. However, the part of phenotypic plasticity and adaptation in these changes is not known.

To our knowledge, there are only three reported cases of evolutionary rescue in vertebrates. In the first one, Trinidadian guppies were experimentally introduced in a new environment with a high predation rate. They evolved an increased ability to escape predators in 26 to 36 generations (O'Steen *et al.* 2002). In the two other studies, native predators (fence lizards and black snakes) were naturally exposed to new toxic preys (fire ants and cane toads

respectively). In the first case, the lizards evolved a behavioral avoidance of the fire ants in 37 generations (Robbins & Langkilde 2012). In the other case, the snakes evolved a behavioral avoidance of cane toads and a physiological resistance to their toxins in less than 23 generations (Phillips & Shine 2006b).

I.1.2. Invasive species and rapid adaptation

I.1.2.1. What is an invasive species?

Definitions of invasive species differ between authors (Valéry *et al.* 2008) but essentially, a species can be considered as invasive if some individuals are introduced outside of their native range, establish self-sustaining populations and spread in the new location (Williamson 1996). According to this definition, different steps are required for a species to become invasive. First the species has to be transported from its native range, then introduced into the wild outside of its native range, survive, and finally, reproduce and spread (figure 6). Introductions can be deliberate as it was the case for game and ornamental species, or accidental like the introductions of rats in harbor with arriving ships. For some authors, species that spread outside of their known native range by diffusion, can also be considered as invasive whereas, for other authors, only exotic species that have an impacts in their introduced range can be considered as invasive (reviewed in Valéry *et al.* (2008)).

The probabilities of crossing the geographic or demographic barriers to go from one of these steps to another are supposed to be quite low but are hard to estimate as only a few introductions present complete enough records. The tens rule is a classic estimation of these probabilities. This rule states that only 10% of the transported species are introduced, from these introduction, only 10% become established and finally, only 10% of established species will spread or become pests (Williamson & Fitter 1996). However, these figures vary across taxa. For example, they are estimated to be much higher in vertebrates and in birds (Jeschke & Strayer 2005). There is probably no general rule but this shows that the establishment of a breeding population in a new environment is not straightforward and requires specific conditions such as a sufficient number of founder individuals and a high enough genetic variability which will allow the species to adapt to the new environment it has been introduced to. Biotic and abiotic characteristics of the ecosystem probably also play a role.

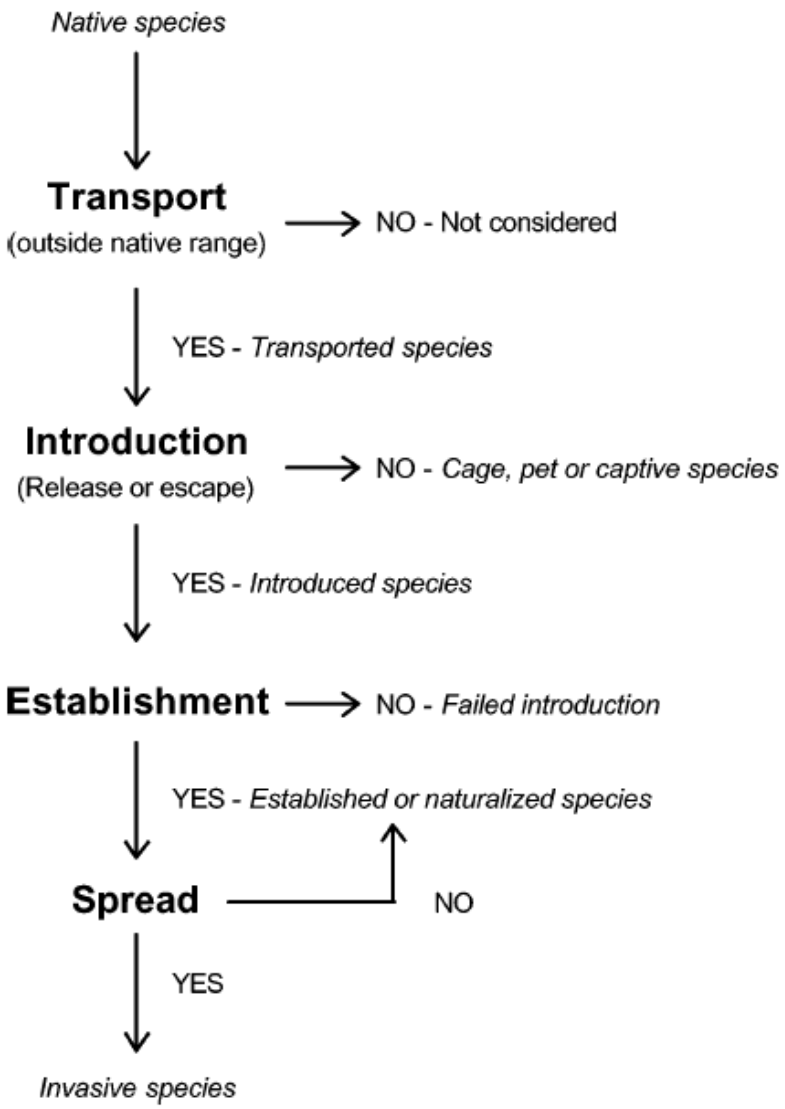

Fig. 6: Steps by which a species goes thought before becoming invasive. From Williamson (1996).

I.1.2.2. Why study rapid adaptation in invasive species?

Biological invasions are the context of 38% of the articles containing the terms "rapid evolution" we found in our search on Web of Science. This highlights the general concerns raised by invasive species and their impacts. Indeed, the number of species introduced outside of their native range has been increasing since the Neolithic in parallel with the augmentation of the frequency and length of human travels, and has reached unprecedented rates with the current frequency of international exchanges (di Castri 1989, figure 7).

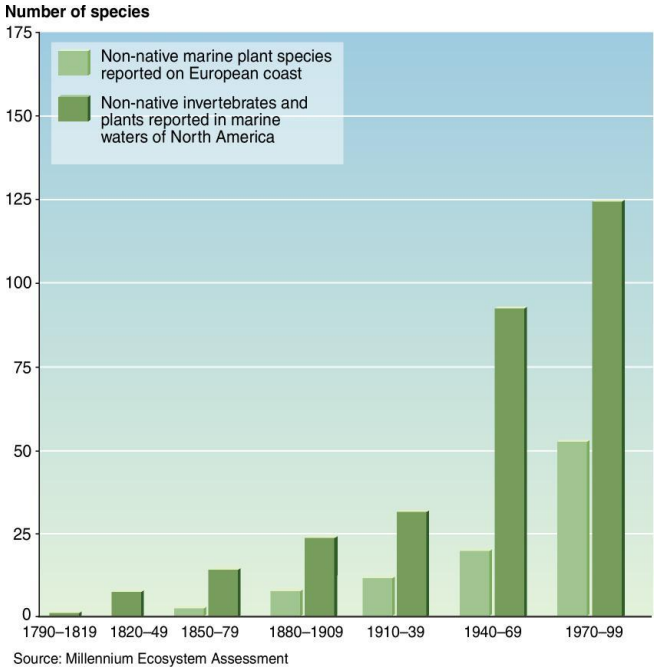

Fig. 7: Evolution with time of the number of invasive marine plant species in Europe, and invasive marine invertebrate species and invasive marine plant species in North America (MEA 2005).

Moreover some invasive species have strong impacts on biodiversity, human health and economy (Vitousek *et al.* 1997; Pimentel *et al.* 2000, 2005). Impact on Biodiversity: the Millennium Ecosystem Assessment report (2005) shows that invasive species are one of the five main drivers of biodiversity loss. For example, in the US, it is estimated that 42% of endangered species are threatened by invasive species (Pimentel *et al.* 2005). There are several mechanisms through which invasive species threaten other species: direct interactions such as predation and parasitism, or indirect interactions such as competition for food or other resources, modifications of ecosystems, and introduction of new parasites. For example, in England, the presence of the grey squirrel which was introduced from the United States has been associated with the decline of the red squirrel. A combination of competition and the transmission of a virus seems to have caused this decline (Sainsbury *et al.* 2000; Gurnell *et al.* 2004). Impact on Human-health: there are many examples of human diseases that are carried by introduced species such as plague epidemics carried by black rats. More recently, Marsot *et al.* (2013) showed that the Siberian chipmunk, a squirrel species introduced in France, is facilitating the spread of Lyme disease as it is a better reservoir than local fauna for this disease (figure 8). Impact on economy: because of the losses, damages and control costs they induce, biological invasions have been estimated to cost about 120 billion dollars per year in the US (Pimentel *et al.* 2000).

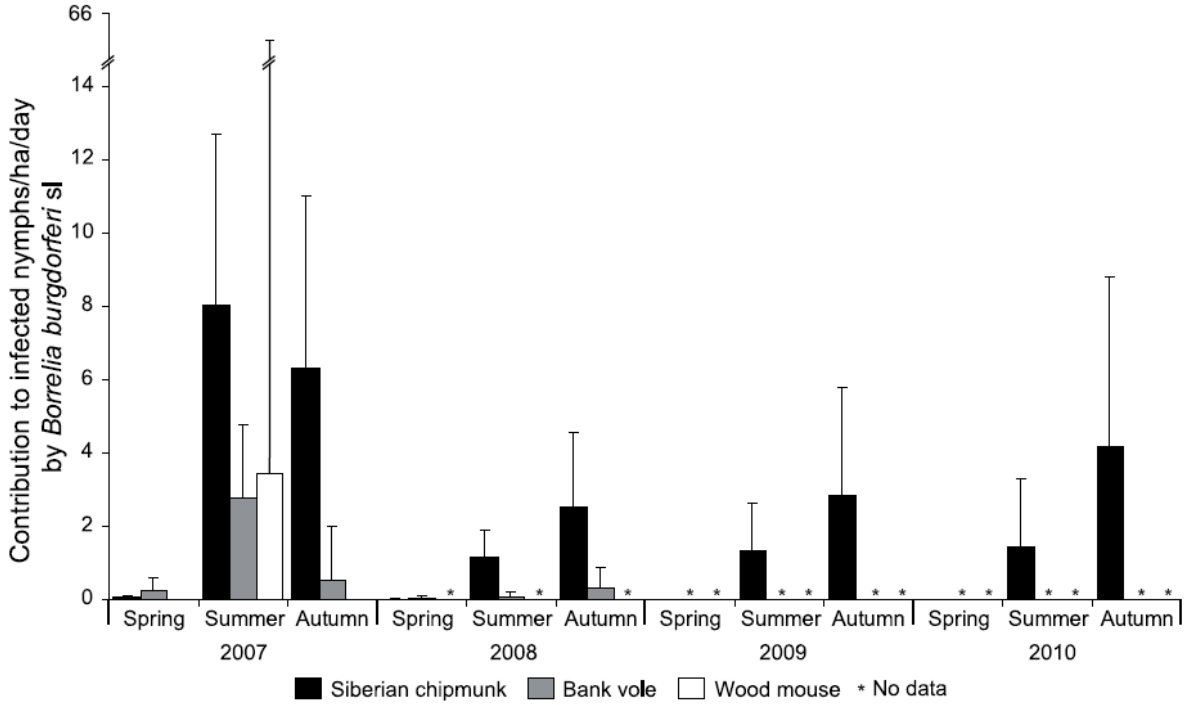

Fig.8: Estimated contributions to Lyme borreliosis (*Borrelia burgdorferi sensu lato*) risk of Siberian chipmunks, bank voles and wood mice. The contributions were estimated for Siberian chipmunks between 2007 and 2010, for bank voles in 2007 and 2008 and for wood mice in 2007, for 3 periods (spring, summer, autumn). Error bars are associated 95% confidence intervals. From (Marsico *et al.* 2010).

There are thus ecological, sanitary and economic reasons for wanting to control invasive species and prevent future introductions. This however requires to understand the characteristics of species and ecosystems that favor invasions, and the processes involved during the invasions (Simberloff 2003; Bacigalupe 2008). Many studies have tried to identify species traits or ecosystem characteristics that can favor invasions but there are few possible generalizations (Case 1996; Kolar & Lodge 2001; Blackburn & Duncan 2001a; b; Cassey 2002). Rapid adaptation has recently been suggested to be a key step in the invasion of a novel environment. Studying its role in biological invasions can thus bring new insights in the way of dealing with invasive species (Sakai *et al.* 2001; Lankau *et al.* 2011). From a theoretical point of view, recently introduced populations also provide a unique opportunity to study evolution in the wild and in real time while individuals are adapting to their new environment.

I.2. What has been done on rapid adaptation, what remains to be done?

In the search we did on Web of Science, we found 274 articles containing the terms "rapid evolution" in the fields of Ecology, Evolutionary Biology and Biodiversity Conservation. About one third of the studies were done on plants, one third on arthropods and one third on vertebrates. Here, I summarized the findings of a part of theses 274 studies. The studies I chose were those done on vertebrates and which showed a rapid phenotypic change observed in a population (table 1). The aim of this summary was to underline the general trends on what is currently done on rapid adaptation but not to exhaustively list all the studies that might exist on this subject.

The changes described in these studies were observed in periods ranging from 150 years to only one year. Time period has a different meaning depending on the generation time of the species considered. However, the generation time of the study species was not always given. Focusing only on articles in which the time was given in number of generations, changes were observed on periods ranging from 3 to 60 generations. The traits that were studied can be classified in four categories: morphological traits, life-history traits, behavioral traits and physiological traits. The suggested causes for these changes were mainly the exposure to a novel environment (most of the studies were on invasive species). Other suggested causes were mortality caused by human activities, changes in climatic conditions and the introduction of a new prey, predator or competitor.

Interestingly, it appears that most of the studies on rapid adaptation we found only report a correlation between a phenotypic change and an environmental change. However, other mechanisms than adaptation can cause phenotypic change: phenotypic plasticity and stochastic evolution (*i.e.* stochastic changes in allele frequencies in a population as the result of demographic processes such as founder effects and bottlenecks). Thus, in order to know if a phenotypic change is due to adaptation, its heritability and its associated increase in relative fitness must be ascertained. In our review, there were only three studies out of 52 which proved these two points. Eight additional studies showed either the heritability or the adaptiveness of the observed phenotypic change.

Table 1: Summary of studies on rapid evolution in vertebrates and which showed a rapid phenotypic change in a population. For each article we give the species studied, the time in which the change was observed in years (y.) or in number of generations (gen.) when this information was available, the traits that changed, the cause suggested by the authors for this change, and we specify whether the heritability and the adaptiveness of the observed change was established.

Species	Time	Traits	Suggested cause	Heritability Adaptiveness	Source
Amphibians					
Bufo marinus	70 y.	body size and toxicity relative to size	novel environment	no	(Phillips & Shine 2005)
Bufo marinus	70 y.	leg length relative to size	segregation in space	no	(Phillips *et al.* 2006a)
Birds					
Carpodacus mexicanus	30 y.	eggshell thickness and pore density	novel environment	no	(Stein & Badyaev 2011)
14 species	100 y.	wing shape	forest management	no	(Desrochers 2010)
Pitangus sulphuratus	17 gen.	body size	novel environment	no	(Mathys & Lockwood 2009)
Geospiza fortis	40 y.	beak size	human altered environment	no	(Hendry *et al.* 2006)
Tympanuchus pallidicinctus	50 gen.	clutch size and number of clutches per year	mortality caused by roads, power lines	no	(Patten *et al.* 2005)
Junco hyemalis	8 gen.	amount of white in the tail	novel environment	yes (heritable)	(Yeh 2004)
Carpodacus mexicanus	20 y.	migratory behavior	novel environment	no	(Able & Belthoff 1998)
Geospiza fortis	1 y.	body size	drought	yes (adaptive)	(Boag & Grant 1981)
Telespyza cantans	20 y.	bill shape	novel environment	no	(Conant 1988)
Geospiza fortis and G. scandens	30 y.	body size and beak shape	climatic variations	no	(Grant & Grant 2002)
Geospiza fortis	22 y.	beak size	new competitor	no	(Grant & Grant 2006)
Pycnonotus jocosus	10 -15 gen.	body and beak shape	novel environment	no	(Amiot *et al.* 2007)
Petrochelidon pyrrhonota	30 y.	wing shape	increased road traffic	no	(Brown & Bomberger Brown 2013)
Turdus merula	60 - 80 y.	migratory behavior	colonization of urban areas	no	(Evans *et al.* 2012)
Fish					
Poecilia reticulata	3 gen.	body size	size-selective harvesting	yes (heritable)	(van Wijk *et al.* 2013)
Gasterosteus aculeatus	8 gen.	size and number of morphological defenses	predation intensity	no	(Leaver & Reimchen 2012)
Coregonus albula	20 y.	rapidity of life cycle	novel environment	no	(Amundsen *et al.* 2012)
Gasterosteus aculeatus	25 y.	body size and diet	novel environment	no	(Adachi *et al.* 2012)
Salmo trutta	20 - 130 y.	body shape	novel environment	no	(Westley *et al.* 2012)
Cyprinella lutrensis	6 - 60 y.	body shape	human altered environment	yes (heritable)	(Franssen 2011)
Coregonus albellus and C. fatioi	25 y.	growth rate	fishing	no	(Nusslé *et al.* 2011)
Gasterosteus aculeatus	3 gen.	cold tolerance	novel environment	no	(Barrett *et al.* 2011)
Perca fluviatilis	20 y.	size at maturation	pathogen outbreak	no	(Ohlberger *et al.* 2011)
Two *Coregonus* species	50 y.	gill raker counts	human-induced eutrophication	no	(Bittner *et al.* 2010)
Poecilia reticulata	30 gen.	female life-history traits	predation intensity	no	(Gordon *et al.* 2009)
Coregonus palaea	25 y.	growth rate	fishing	no	(Nusslé *et al.* 2009)
Salvelinus alpinus	6 gen.	body shape and size at maturity	novel environment	no	(Michaud *et al.* 2008)
Cyrpinodon tularosa	30 y.	body shape	salinity	yes (adaptive)	(Collyer *et al.* 2007)
Lepomis macrochirus	40 y.	feeding morphology	novel environment	no	(Yonekura *et al.* 2007)
Poecilia reticulata	13 - 26 gen.	color	predation intensity	yes (heritable)	(Karim *et al.* 2007)
Cynotilapia afra	40 y.	color	novel environment	no	(Streelman *et al.* 2004)
Gasterosteus aculeatus	20 y.	number of pectoral fin rays	novel environment	no	(Kristjánsson *et al.* 2004)
Gasterosteus aculeatus	13 y.	number of spines and armor plates	novel environment	no	(Kristjánsson *et al.* 2002)
Poecilia reticulata	26 - 36 gen.	escape abilities	predation intensity	yes (heritable and adaptive)	(O'Steen *et al.* 2002)

Gambusia affinis	55-58 y.	size at maturity and fat content	novel environment	yes (heritable)	(Stockwell & Weeks 1999)
Poecilia reticulata	4 - 11 y.	age and size at maturation	predation intensity	no	(Reznick 1997)
Oncorhynchus tshawytscha	30 gen.	freshwater growth rate, phenology	novel environment	no	(Quinn *et al.* 2001)
Coregonus albula	10 y.	growth rates, fecundity and size at maturation	novel environment	no	(Bøhn *et al.* 2004)
Mammals					
Pseudocheirus peregrinus	60 gen.	anti-predator behavior	new predator	no	(Anson & Dickman 2013)
Peromyscus leucopus	25 y.	morphology	human altered environment	no	(Pergams & Lacy 2008)
Peromyscus maniculatus	90 y.	morphology	Unknown	no	(Pergams & Ashley 1999)
Three *Rattus* species	150 y.	skull size	novel environment and competition	no	(Yom-Tov *et al.* 1999)
Reptiles					
Sceloporus undulatus	37 gen.	feeding preference	new toxic prey	yes (heritable and adaptive)	(Robbins & Langkilde 2012)
Anolis cristatellus	35 y.	low-temperature tolerance	new climate	no	(Kolbe *et al.* 2012)
Malaclemys terrapin	10 - 20 gen.	female growth rate and body size	size-selective mortality	no	(Wolak *et al.* 2010)
Gloydius blomhoffii	20 gen.	body size, life-history traits, and antipredator behavior	hunting	yes (heritable)	(Sasaki *et al.* 2009)
Anolis cristatellus	1 y.	skin resistance water loss	drought	no	(Perry *et al.* 2000)
Anolis sagrei	10 - 14 y.	hindlimb length relative to size	novel environment	no	(Losos *et al.* 1997)
Pseudechis porphyriacus and *Dendrelaphis punctulatus*	20 gen.	body and gape size	new toxic prey	no	(Phillips & Shine 2004)
Pseudechis porphyriacus	23 gen.	feeding behavior and toxin resistance	new toxic prey	yes (heritable and adaptive)	(Phillips & Shine 2006b)

In conclusion, we see that there are already a fair number of studies highlighting phenotypic changes that might be caused by rapid evolution but there is a lack of information on the basic mechanisms causing these changes. This lack of evidence probably partly arises from the difficulty of demonstrating adaptation (Merilä & Hendry 2014). Indeed, classical ways of testing adaptation are common garden experiments, reciprocal transplants and animal models (Merilä & Hendry 2014). However, common gardens experiments require the raising of wild individuals in captivity and this is not always possible for practical or ethical reasons. Data acquisition following reciprocal transplants requires the follow up of individuals transplanted and their offspring, and there are also legal and ethical problems associated with the transplantation and release of some species into the wild. Finally, animal models allow assessing the heritability of a trait but require data on many individuals with known pedigrees. Long term follow-ups of populations with mark and recapture methods are thus needed to use these models (*e.g.* Charmantier et al. 2008).

We therefore argue that alternative ways to assess if a phenotypic change is caused by rapid adaptation are needed. This would enable the scientific community to accumulate evidence of rapid adaptation and to study its role in biological invasions and its potential in the rescuing of species threatened by global changes.

I.3. Question and methodological choices

In this thesis, the question we wanted to address was: **can rapid adaptation explain phenotypic changes observed in introduced populations?** Indeed, we have seen that phenotypic changes have been reported in invasive populations and knowing if rapid adaptation can explain these changes would be useful in the management of these species (Lankau *et al.* 2011). Moreover, finding evidence of rapid adaptation to an abrupt environmental change can also have implications in the conservation of endangered species as this would mean that evolutionary rescue can occur. However, as we have seen before, demonstrating adaptation in a natural system is often long and is not possible for all species. This is probably one of the reasons explaining why studies on rapid adaptation often only report a correlation between a rapid phenotypic change that seems adaptive and an environmental change.

Instead of directly testing the hypothesis of rapid adaptation to explain rapid phenotypic changes in introduced populations, we thus chose to test for alternative hypotheses. A phenotypic change can be caused by natural selection but also by phenotypic plasticity and by non-adaptive, or stochastic, evolution (*i.e.* stochastic changes in allele frequencies in a population as the result of demographic processes such as founder effects and bottlenecks). Phenotypic differences observed between several introduced populations can also be caused by the fact that they have different phylogenetic origins or mixed origins, and thus different genetic background. Testing for these alternative hypotheses can be easier than directly testing for adaptation and allows identifying cases of rapid adaptation when they can be rejected. However, one must bear in mind that all these mechanisms are not exclusive and that they can act in parallel. Thus, showing an effect of one of these mechanisms does not mean that the others are not involved in the phenotypic changes observed.

Here, we chose to work on two complementary species that are both successful invasive species. We tested the hypotheses of a difference in origin and of a stochastic evolution to explain rapid phenotypic changes we observed. The aim was to assess if these hypotheses could explain the rapid phenotypic changes observed. If not, this would indicate that phenotypic plasticity and/or rapid adaptation are the cause of these changes.

Among phenotypic traits we could have studied, we chose to study the morphology of individuals, and in particular the beak shape. Indeed, morphological traits are known to evolve with changes in environmental conditions. For example, beak shape has been showed to evolve with changes in diet (*e.g.* Boag and Grant 1981; Herrel et al. 2005), and wing shape

with changes in vegetation (Desrochers 2010). In general, body size and shape is correlated to the climatic region inhabited by species (Bergmann's rule, (Bergmann 1847)). Moreover, morphological traits are believed to be highly heritable in birds (Boag 1983; Smith and Dondt 1980). Thus, the effect of phenotypic plasticity can be expected to be relatively low in morphological changes. Finally, morphological measurements can be obtained from collection specimens contrary to behavioral or physiological traits, for example. This allowed us to include morphological data from museum specimens in our study and too increase significantly our sample sizes.

Our approach consisted in four steps: (1) comparing morphological traits between individuals of populations introduced in different environments to identify phenotypic differences between some of them; (2) assessing the phylogenetic origin of these populations with a phylogeographic study, and comparing morphological traits between individuals of introduced populations and their source to investigate whether phylogenetic origin can explain the differences observed between populations; (3) assessing the role of the recent demographic history of introduced populations on their morphological differentiation with the study of neutral genetic loci; (4) when it was possible, we also assessed the repeatability of morphological changes by comparing the morphological differentiation observed in replicated situations (*i.e.* populations with the same phylogenetic origin, introduced at similar periods and in similar environments). Indeed, we considered that if a phenotypic differentiation was repeatable, it was likely to be adaptive.

I.4. Presentation of our two case studies

I.4.1. The Red-whiskered bulbul (*Pycnonotus jocosus*)

I.4.1.1. Native range and introduction history

The Red-whiskered bulbul is a passerine bird native from South-East Asia (Peters 1960). Nine subspecies of Red-whiskered bulbul have been described in its native range, based on coloration patterns and morphology (del Hoyo et al. 2005, figure 9). The Red-whiskered bulbul is a popular cage bird and has been introduced accidentally in several regions of the Pacific and Indian oceans, where it became established: the United States

(California, Florida and Hawaii), Australia (New South Wales and South Australia), Mauritius, Seychelles, Comoros islands, and Reunion (Lever 2010).

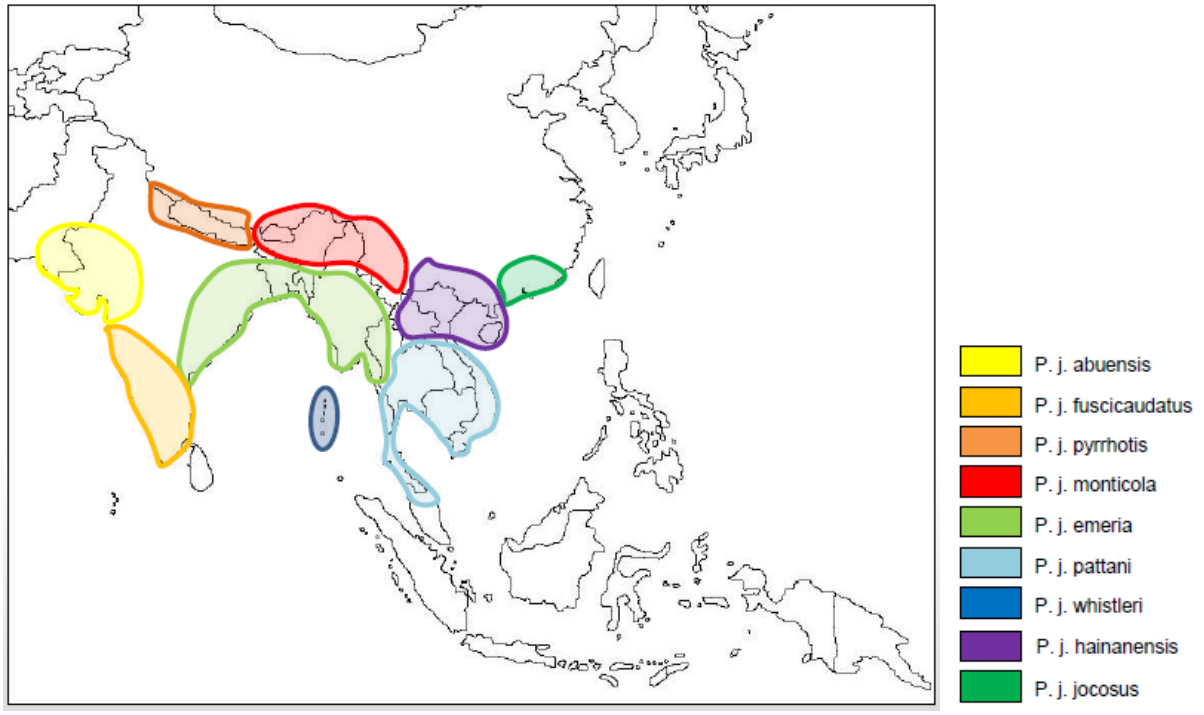

Fig. 9: Native ranges of the nine sub-species of Red-whiskered bulbul as described in Peters (1960).

I.4.1.2. Interesting characteristics for our study

Large populations of Red-whiskered bulbuls have established in most places where this species has been introduced. If, as mentioned earlier, a capacity of rapid adaptation is truly a factor explaining the establishment success of some invasive species, one might expect to find signs of rapid adaptation in invasive populations of Red-whiskered bulbuls. Moreover, a rapid morphological divergence has already been reported in two populations of Red-whiskered bulbuls introduced in Reunion Island and living in different environments (Amiot *et al.* 2007). As suggested by the authors of this study, this could be the result of a rapid adaptation to contrasted environments. Indeed, Reunion Island is separated in two by a high mountain range which creates a difference in climatic conditions between the two coasts. The windward side is exposed to the prevailing wind and the clouds form on this side of the mountains. It is thus more humid than the leeward side which is protected of the wind by the mountains (figure 10). This difference in humidity between the two sides generates differences in vegetation and environmental conditions.

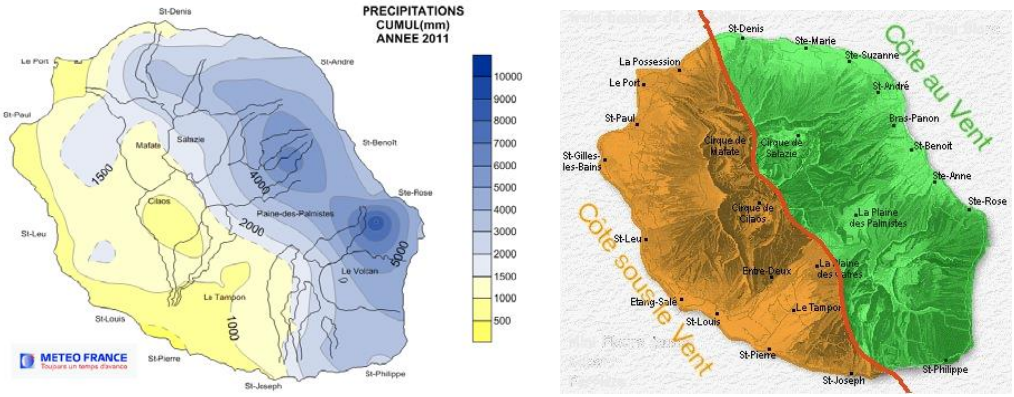

Fig. 10: Left: annual cumulated rainfalls in millimeters on Reunion Island for 2011; from Météo France. Right: delimitation of Reunion windward (green) and leeward sides (orange).

Moreover, what is known from the literature on the introduction of Red-whiskered bulbuls on Reunion Island is that they have been introduced in the South-east of the island in 1972. From this point, they colonized the two coasts and the two populations are believed to have been isolated since then because of the mountain range (Clergeau & Mandon-Dalger 2001). The study of Amiot *et al*. (2007) was based on 11 morphological measurements taken on 272 individuals that were caught in 13 sites spread along the coasts. The authors used a clustering approach to identify morphological groups. They found morphological differences between bulbuls from the two coast and this difference was significant when beak measurements were considered alone (figure 11). It is therefore possible that bulbuls adapted to contrasted local conditions on Reunion in only a few generations (between 15 and 20).

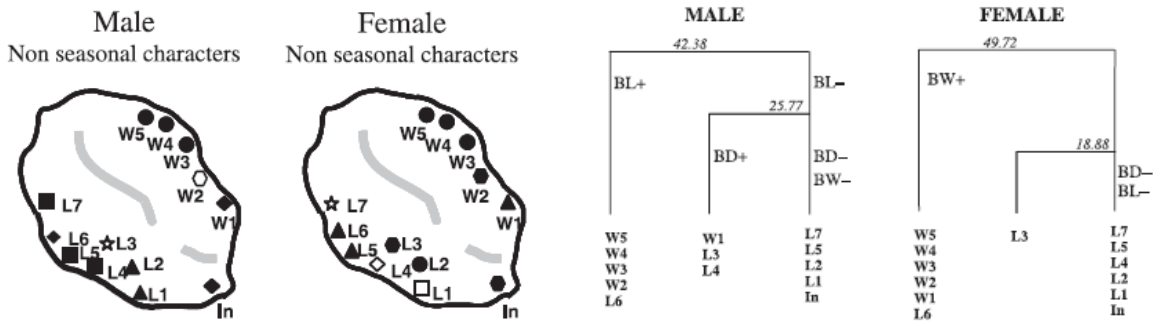

Fig. 11: Left: Map of Reunion Island showing the classification of sampling sites according to morphological measurements. Right: Classification of sites according to beak measurements. Adapted from (Amiot *et al.* 2007).

Finally, Red-whiskered bulbuls have been introduced on other tropical islands sharing the same geographic characteristics than Reunion Island. The study of the morphology of bulbuls on these islands could thus be used to assess if the morphological divergence observed on Reunion is repeatable and thus if it is likely to be adaptive.

I.4.1.3. Sampling

In order to study the effect of historical factors on morphological changes in populations of Red-whiskered bulbuls, we studied the same individuals as those used in Amiot *et al.* (2007), and we collected additional samples on two other islands: Mauritius and Oahu (Hawaiian archipelago). The Red-whiskered bulbul has been introduced on Mauritius, probably from India in 1892 (Lever 2010). From there, it was introduced on Reunion in 1972 (Lever 2010). The colonization of Reunion by the Red-whiskered bulbul is relatively well documented. It was introduced at the south-eastern point of the island. The population rapidly expended from there, first on the humid and forested windward coast during the 1980's and then on the drier leeward coast during the 1990's (Clergeau & Mandon-Dalger 2001). Oahu was colonized at the same period as Reunion (1965) but from an unknown source (Lever 2010). These three islands have similar sizes and present windward and leeward coasts with contrasted environments.

On Reunion, 437 bulbuls were caught at 12 sites along the coasts in 2002-2003 during a control program organized by the FDGDON (Fédération Départementale des Groupements de Défense contre les Organismes Nuisibles de la Réunion). In 2013, we collected data on 50 individuals in 3 sites on Mauritius. There was one site on the windward coast and two sites on the leeward side. Forty five individuals were also caught the same year on Oahu in two sites, one on each side of the island (figure 12).

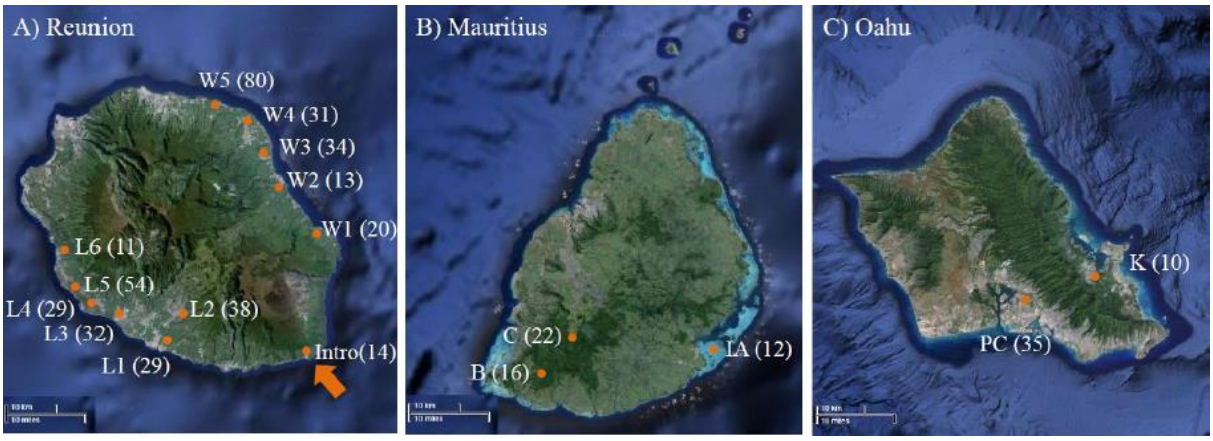

Fig. 12: Study sites on Reunion, Mauritius and Oahu (Hawaii). The number of individuals caught at each site is in brackets. The arrow shows the point of Red-whiskered bulbul introduction on Reunion.

I.4.2. The Ring-necked Parakeet (*Psittacula krameri*)

I.4.2.1. Native range and introduction history

The Ring-necked parakeet (*Psittacula krameri*) is native from the Indian subcontinent and sub-Saharan Africa where it mainly lives in warm climates. In Asia, its range however reaches the base of the Himalayas (up to 1600 m, Parr and Juniper 2010) indicating a tolerance for colder climates (Thabethe *et al.* 2013). It is found in a variety of woodlands but also in savanna grassland, farmland, and parks and gardens in urban areas (Parr & Juniper 2010). Four sub-species have been described, two in Asia and two in Africa, based on color and size differences (del Hoyo et al. 1997, figure 13). The Ring-necked parakeet is a popular cage bird and has been introduced accidentally in many countries since the 1960s. In Europe, populations of Ring-necked parakeets became established in Belgium (1966), the Netherlands (1968), Great Britain (1969), Germany (1969), France (1970s), Italy (1970s), Spain (1982), Portugal (1986), and Greece (1992, Braun 2009).

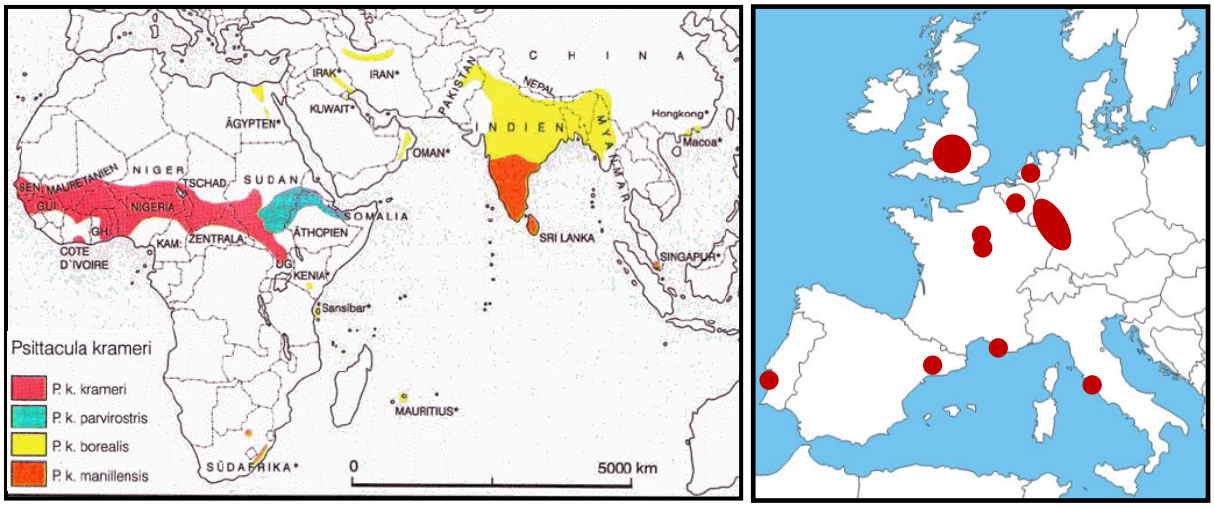

Fig. 13: Left: native ranges of the four Ring-necked parakeet subspecies. From (Strubbe 2009). Right: location of established populations of Ring-necked parakeets in Europe. After (Braun 2009).

The origin of these introduced populations is not clearly established. Morphological measurements of individuals caught in the United Kingdom suggest that introduced individuals are more similar to the Asian subspecies *P. k. borealis* whereas their beak coloration suggests a closer relationship to the Asian subspecies *P. k. manillensis* (Butler 2003). However, CITES data show that Ring-necked parakeets have been imported from Asia and Africa in comparable proportions (CITES 2013, table 2).

Table 2: Number of Ring-necked parakeets imported in European countries from Asia and Africa between 1985 and 2010 according to the CITES data base.

Importing country	Importations from Africa Counts and proportion of total	Importations from Asia Counts and proportion of total	Total counts
Italy	27 955 - 29%	69 766 - 71%	97 721
Portugal	41 087 - 85%	7 010 - 15%	20 522
Spain	27 007 - 64%	15 250 - 36%	42 257
UK	15 278 - 65%	8 145 - 35%	23 423
France	17 568 - 86%	2 974 - 14%	20 522
Germany	13 211 - 67%	6 597 - 33%	19 808
Belgium	6 213 - 65%	3 379 - 35%	9 592
Netherlands	4 815 - 50%	4470 - 50%	9585

I.4.2.2. Interesting characteristics for our study

Successful populations of Ring-necked parakeets have settled in Europe despite their tropical origin. We thus hypothesized that the Ring-necked parakeet had to adapt to these new climatic conditions following its introduction. Moreover, as the Red-whiskered bulbul, it is a very successful invasive species. It therefore seemed a good model species to study rapid adaptation. In addition, European Ring-necked parakeet populations are present in three type of climate: Mediterranean, oceanic and semi-continental. This was likely to increase our chances to find adaptive phenotypic differences between populations. Finally, all the populations where introduced at about the same time and some share the same climate. The comparison of 'replicate' populations in similar situations was thus possible.

This species is complementary to the Red-whiskered bulbul for our study. Indeed, contrary to the bulbul, it has been introduced in places that are both different from the native range and that are different from each other. However, it also has its limits as morphological differences had never been observed between populations before this thesis. We were thus unsure that there would be any differences. Furthermore, we knew that sampling would be difficult because parakeets are difficult to catch and because other research groups were already working on almost every population established in Europe.

I.4.1.3. Sampling

In order to assess whether the Ring-necked parakeet has evolved since its introduction in Europe, we collected data from locations with comparable climate (two populations close

to Paris, France) and from locations in different climatic regions (one population in Barcelona, Spain and one in Heidelberg, Germany). Ring-necked parakeets have been introduced there in 1974, 1982 and 1990 respectively. The population of Heidelberg is younger than the others but it was founded by individuals which dispersed from Neckarhausen where parakeets were introduced in 1974 (Braun 2009). We thus considered that our four study populations had about the same age. We also considered that the populations of Ring-necked parakeets around Paris had been founded independently. Indeed, yearly follow-ups of breeding sites showed that the parakeets expended from two distinct centers (Clergeau *et al.* 2009). Each of these centers is close to one of the Parisian airports where the parakeets were probably released accidently from planes (figure 14). Before this thesis, no information existed on the connectivity between these two populations.

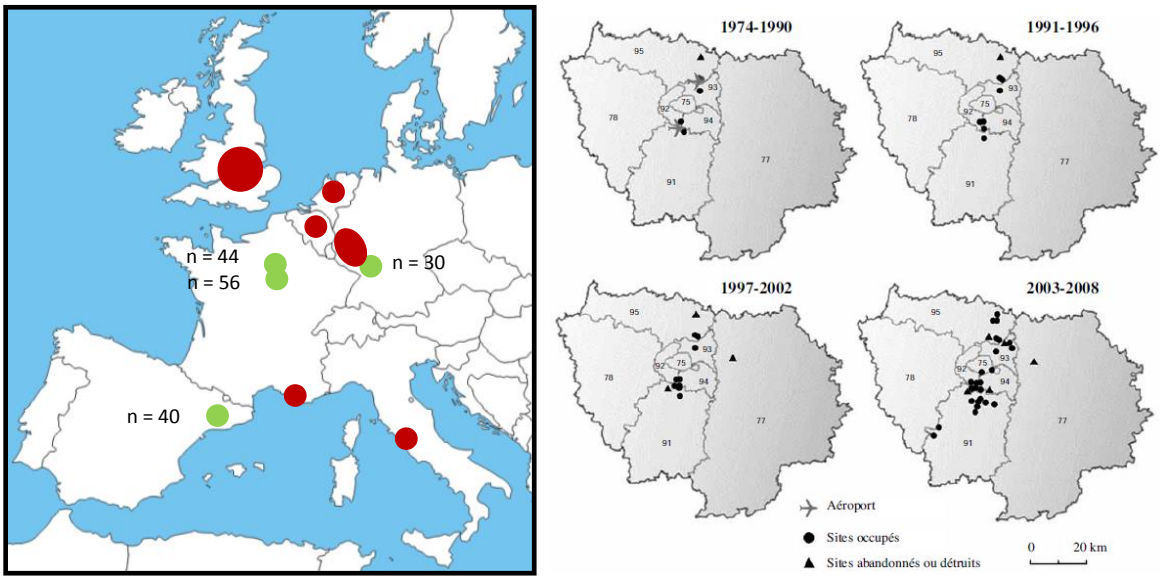

Fig. 14: Left: Study sites of Ring-necked parakeets (green dots), sample size are showed beside each point. Right: Spread of Ring-necked parakeet in Ile-de-France based on their breeding sites. From (Clergeau *et al.* 2009).

II. Methods

II.1. Sampling

At the beginning of my PhD, only Red-whiskered bulbuls from Reunion were already available at the lab, left from a previous study. As part of my project, I collected data on Red-whiskered bulbuls in other introduced populations and in the native range. The study on the Ring-necked parakeet is a totally new project and I collected the whole dataset for this species. I organized field missions to catch bulbuls in Oahu (Hawaii) and Mauritius and to catch parakeets in Paris and Marseille. I also established collaborations and visited museum collections to complete my sampling.

II.1.1. Development of capture protocols

In Oahu, I caught Red-whiskered bulbuls with mist nets in private gardens were they were used to get fed. In Mauritius, I caught them in aviaries built up to feed protected endemic birds and in which they were used to come to get food too. All the individuals caught were marked, measured, photographed in standardized conditions and released immediately afterwards. From each individual, two feathers were collected for genetic analyses. Feathers were stored frozen at -20°C.

Ring-necked parakeets preferentially remain in the canopy of tall trees and rarely come close to the ground, making mist nets not suitable for their capture. During the first year of my PhD I tested different kinds of way to catch them. The sites I chose were private gardens where parakeets were known to visit bird feeders regularly. These sites were found either by following parakeets after they had left their roosting sites in the morning or thanks to advertisements in local journals.

The best devices to catch the parakeets were magpie traps positioned at about 1.5 meters above the ground (figure 15). This kind of trap closes automatically when the birds step into it and is composed of four compartments. Wooden perches were fixed at the entrance of each compartment to enable parakeets to enter. Sunflower seeds and peanuts were placed inside the traps to attract parakeets. The cages were left on the sites during the whole winter season as parakeets visit bird feeders every day in winter. The traps were blocked in open position so that parakeets could get used to feed inside. Once per week, the traps were unblocked before the arrival of parakeets and all the parakeets caught during the morning were measured. Parakeets were very suspicious toward traps. Sometimes it took several

weeks before they started entering into the traps and after a capture session several days might pass before they come back. Two other kind of traps were tested but were less efficient as they had to be closed manually and parakeets were reluctant to enter into the traps when they detected human presence. Moreover, the traps for magpie had the advantage that parakeets were attracted to the traps if one parakeet was already inside. As there were four compartments, we could catch several parakeets at a time.

Initially, we had planned to catch parakeets in the North of Paris, in the South of Paris and in Marseille. The population of Marseille would have been used as a replicate for the population of Barcelona. However, parakeets in Marseille were much more suspicious towards traps than in Paris and were never observed to enter into them.

Like for bulbuls, all the individuals caught were, marked with a metal ring, measured, photographed in standardized conditions and released immediately afterwards. Two feathers were collected on them for DNA extraction. Feathers were stored frozen at -20°C.

Fig. 15: A Ring-necked parakeet inside one of the compartments of a magpie trap placed on a pole.

II.1.2. Collaborations

In order to get data from supplementary introduced populations of Ring-necked parakeets, I established collaborations with Dr. Juan Carlos Senar (Museu de Ciencies Naturals de Barcelona, Spain) and Dr. Michael Braun (Heidelberg University, Germany). They provided me DNA samples and pictures from Ring-necked parakeets captured in Barcelona and Heidelberg. For the field work in the Hawaii, I collaborated with Dr. Blake Matthys (Ohio Dominican University, United States) to carry out the capture of Red-whiskered bulbuls in Oahu. Finally, I visited the collections of the Muséum National d'Histoire Naturelle (MNHN) and the British Natural History Museum (BNHM) to collect morphological data (measurements and pictures) on specimens from the native range of both species. I also obtained tissue samples on loan from several museums for the phylogeographic analyses of both species: Muséum National d'Histoire Naturelle (Paris), British Natural History Museum (Tring), Field Museum of Natural History (Chicago), University of Michigan Museum of Zoology (Ann Arbor), California Academy of Sciences (San Francisco), Australian Museum (Sydney) and Museum Victoria (Melbourne).

II.2. Morphological comparisons

The aim of this part was to compare the phenotype of individuals between populations in order to assess if a phenotypic differentiation has occurred in some introduced populations. As explained in the introduction, the phenotypic traits chosen were some morphological characters that were expected to vary across environments and that could be measured on both live individuals and museum specimens. We used two complementary methods to describe the morphology of individuals: classical measurements and geometric morphometrics. The advantage of classical measurements is that they are obtained rapidly and that they can be taken on every parts of the body. However, some studies show that museum specimens tend to shrink with time and thus there is a bias in the lengths measured (reviewed in Engelmoer *et al.* 1983; Eastham *et al.* 2000). In addition, the different parts of the body do not shrink with the same amplitude. Some correction factors exist in the literature but they are very dependent on the species, on the trait considered and on the age of the specimens. As there are no correction factors available for *Psittacidae*, we decided not to use any corrections but rather to bear in mind that there was a possible bias in our data because some individuals were measured alive while some others were measured as skins. Using museum specimens was our

only way to obtain data from the native ranges of our two model species. Indeed, organizing field missions to Asia and Africa would have been too costly and time-consuming in the framework of a PhD thesis. In order to reduce the bias caused by the comparison of live individuals and museum specimens, we decided to also use geometric morphometrics to describe the morphology of our specimens. With this approach, it is the conformation of the body that is studied and not lengths. We hypothesized that this measure would be less sensitive to specimen shrinkage. Furthermore, we decided to work on the beak of the individuals as some authors suggest that it shrinks less than other parts of the body such as wings and tail (*e.g.* Engelmoer *et al.* 1983). However, this approach had its drawbacks as the digitization process is very long and it allowed us to work only on a small part of the body. Indeed, it would have been very difficult to work on other parts such as wings for example as this technique requires that the organ studied is photographed in standardized conditions for every specimen. It would have been impossible to study the wings of museum specimens as they are not always spread in the same way from one specimen to the other. This is why we used both classical measurements and geometric morphometrics to describe the morphology of individuals.

II.2.1. Data acquisition

The measurements chosen had to be measurable both on live individuals and museum specimens and therefore the tarsus length was excluded. All the other classical measurements on the beak and the body were taken. Beak length, beak width, beak depth and cranium length were measured with a digital caliper (to nearest 0.1 mm; figure 16). Folded wing length and central tail feather length were measured with a metal ruler (to nearest 0.5 mm). All measures were taken by me and to increase precision, they were taken twice and averaged for live individuals.

For the geometric morphometric analysis, I chose to collect data on the beak as it was a part of the body that could be photographed in standardized conditions without too much bias. Pictures were taken in lateral view in standardized conditions (*i.e.* orthogonally, on a white background, with a scale, approximately from the same distance and with the same camera settings). It was not possible to take pictures on dorsal view of museum specimens and this view was abandoned. Points were digitized from these pictures with TPSDIG 2 (Rohlf 2010a) in order to describe the beak shape. Landmarks placed on homologous points (*i.e.* points identifiable on all individuals), and semi-landmarks equally spaced and describing

outline curves, were digitized (figure 16). All pictures were digitized by me and the repeatability was tested for both species using principal components analyses (PCA) on three repetitions taken on five specimens chosen randomly from the same sampling site.

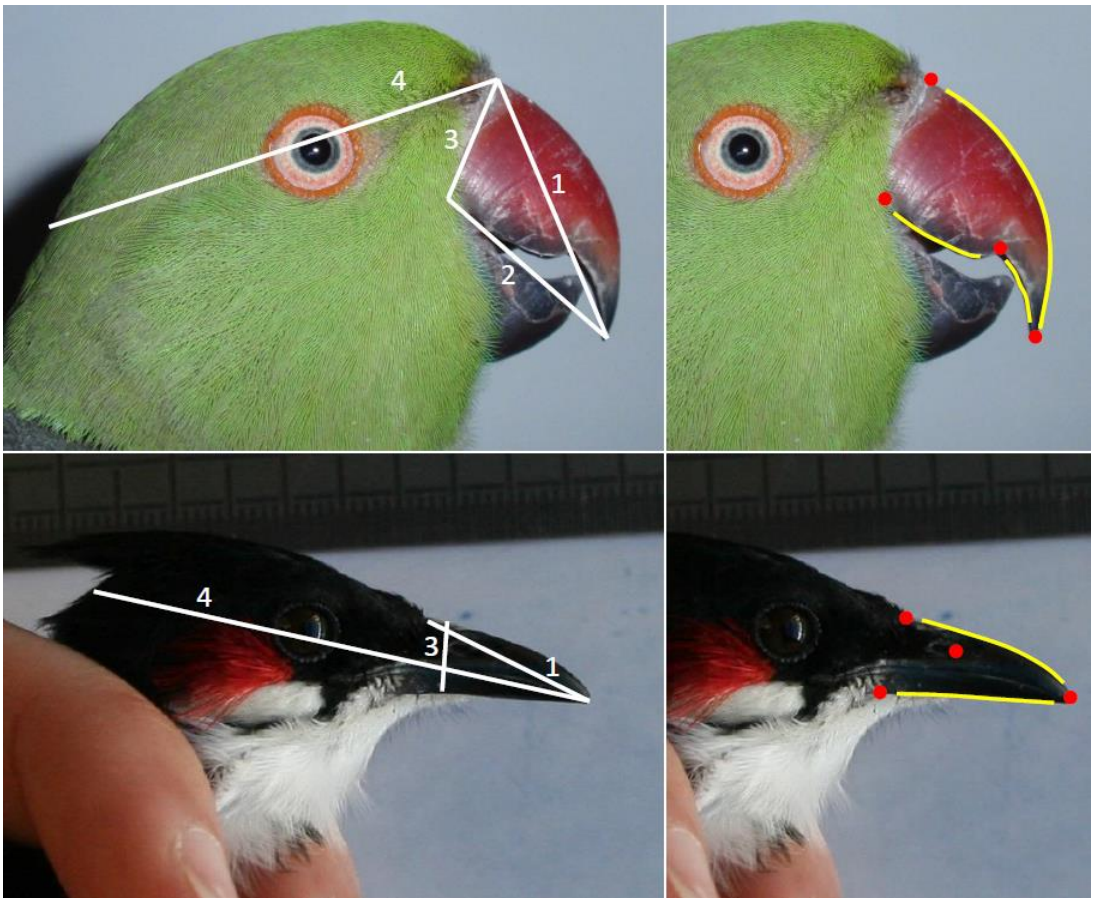

Fig. 16: Left: classical morphological measurements taken on the head of parakeets (upper part) and bulbuls (lower part); 1: beak length, 2: second beak length (only measured on parakeets), 3: beak depth, and 4: cranium length (including beak for bulbuls). Right: Landmarks and outline curves digitized on pictures of the beak in lateral view for Ring-necked parakeets (upper part) and Red-whiskered bulbuls (lower part).

II.2.2. Data standardization

Classical morphological measurements were transformed into log-shape ratios in order to control for the size effect on the body parts measured (Mosimann & James 1979). Following this method, the overall size of each individual was defined as the mean of the log-transformed measurements. Each measurement was then standardized by subtracting the overall size of the individual to the log-transformed measured value.

A Generalized Procrustes superimposition (Rohlf & Slice 1990) of the points digitized for each individual was performed using TPSRELW (Rohlf 2010b). With this method the set

of landmarks digitized for each individuals are transformed in order to minimize differences between individuals. This is done by adjusting their position, rotation and scale while conserving the shape they define (Adams *et al.* 2004). The size information is thus removed. Semi-landmarks are also slid along the curves they describe to match as well as possible the positions of the corresponding points in a reference specimen randomly chosen (Adams *et al.* 2004). The coordinates obtained after this step were those used for the analyses of beak shape. The size of the beak of individuals was defined as the log-transformed centroid size (square root of the sum of the square distances between the landmarks and their centroid).

II.2.3. Analyses

Log-shaped ratios describing the whole body and Procrustes residuals describing the beak shape were analyzed separately. PCAs were used to summarize the information contained in the data sets with fewer variables. The principal component scores representing 95% of the total variance were kept as morphological variables for further analyses. The "centroid individual" of each source and introduced population was defined (mean coordinates on each axe) and plotted in the morphospaces defined by the different axes to see the differences between populations. Neighbor-joining trees based on Euclidian distances between centroids were also constructed to visualize the differences when all axes were considered together. Multivariate regressions were performed between size and morphological variables to test for allometric effects. Multivariate analyses of covariance (MANCOVAs) were then performed to assess if there was a significant effect of population membership on morphology. Sex was added as co-factor to control for sexual dimorphism. Size was not added as it was always correlated to sampling sites. Size was not investigated further as it did not show any specific pattern. In order to investigate the differences between pairs of populations, Hotelling T-squared tests were used and the threshold of acceptance of the null hypothesis was divided by the number of pairwise comparisons performed following the Bonferonni correction.

II.3. Phylogeography

The aim of this part of my thesis was to identify the source of the different introduced populations we studied in order to assess the effect of the phylogenetic origin of individuals on their morphology.

II.3.1. Choice of sequences and amplification protocols

The sequences chosen for the analyses had to be variable enough to enable the discrimination of the different populations. For nuclear DNA, genes are generally too conserved at this scale and could not be used. Unfortunately, little genetic data is available on my two model species and we could not find introns variable enough to be used. I amplified the flanking regions of the microsatellite loci used in the population genetics study but they were also too conserved in my samples. The phylogeographic analyses are therefore based on mitochondrial sequences only. For both species, genes for which there were sequences deposited on Genbank were chosen to complete the sampling. The cytochrome oxidase subunit I (COI) and the NADH dehydrogenase II (ND2) were used for the Red-whiskered bulbuls, and the cytochrome *b* (Cytb) for the Ring-necked parakeets.

DNA extraction and amplifications were done using classical protocols except for museum specimens for which DNA was too fragmented to amplify genes in one piece. In these cases, genes were amplified in small overlapping fragments of 200-300 bp.

II.3.2. Analyses

The nucleotide substitution models for the genes we studied (*i.e.* models describing the different probabilities of change from one nucleotide to another) were selected with MRMODELTEST 2.3 (Nylander 2004) in association with PAUP* (Swofford 2003). MRMODELTEST allows the comparison 24 models of nucleotide substitution. These models combine different parameters describing the frequencies of each nucleotide in the sequence, the substitution rate of each nucleotide, the proportion of invariable sites, and the heterogeneity of the rate of substitution among sites (Posada & Crandall 2001). The models are compared using the Akaike Information Criterion (Akaike 1974) which quantifies the fit of the model to the data. Phylogenetic trees were the inferred with MRBAYES 3.1.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) using the nucleotide substitution model chosen with MRMODELTEST and uniform prior distributions for trees topology and branches length. This software estimates the posterior probabilities of trees and branches length using Markov chain Monte Carlo sampling which explores the "landscape" of parameters combinations in an iterative way. At each step, the chain choses its next step among several possibilities by selecting the one with the highest fit to the data. If the chain is run long enough, the best combination of parameters can be approached.

A complementary approach using maximum likelihood was also used to infer phylogenetic trees in order to compare the trees obtained with these two methods.

II.4. Population genetics

The aim of this last part was to assess the role of stochastic evolution on the phenotypic differences observed between populations. Indeed, a loss of genetic diversity is expected in introduced populations because of potential bottlenecks and founder effects (*i.e* stochastic sampling of the genetic diversity found in the source population, at the moment of the introduction). On the other hand, admixture between populations introduced from different sources can create new genetic combinations (Facon *et al.* 2008). This might affect the phenotype of individuals at the introduction and, afterwards, its evolution (Barrett & Schluter 2008).

II.4.1. Choice of neutral genetic markers

In order to study the demographic history of a population, neutral genetic markers are needed (*i.e.* loci that are not under selection and that can thus track other evolutionary forces). We chose to use microsatellite loci as they are generally highly polymorphic. This was indeed necessary for the study of recently introduced populations. The regions flanking the microsatellites have also the advantage of being usually conserved across species. Thanks to this property, we were able to use primers developed in species closely related to our models. Finally, microsatellite loci can be multiplexed which reduces the cost of amplification and genotyping.

For both species, I tried to amplify an important number of microsatellite loci found in the literature on a small subsample of individuals from different populations. Only the loci that amplified well and that were polymorphic were kept. In the end I retained ten polymorphic microsatellite loci for the Red-whiskered bulbul and 18 for the Ring-necked parakeet. These loci were then amplified for all individuals in several multiplex and tagged with fluorescent forward primers. Genotyping was done at the lab on an Applied Biosystems 3130XL DNA sequencer. Genotypes were scored with GeneMapper 4.0 (Applied Biosystems) and checked manually.

II.4.2. Analyses

FreeNA (Chapuis & Estoup 2007) was used to detect the presence of null alleles. Indeed, null alleles can bias the estimations of F_{ST} and genetic distances (Chapuis & Estoup 2007). When loci with null alleles were found, we either used corrected F_{ST} when possible or we removed these loci from the analyses.

A clustering approach based on Bayesian computation and implemented in the software STRUCTURE 2.3.3 (Pritchard *et al.* 2000; Falush *et al.* 2003) was then used to describe the genetic structure in the introduced populations of Red-whiskered bulbuls and Ring-necked parakeets. The principle of this approach is to assign individuals based on their genotypes to a defined number of clusters which are characterized by a set of allele frequencies at each locus. The individuals are assigned so that loci are at Hardy-Weinberg equilibrium, and linkage equilibrium within clusters. The assignation is done with a Markov chain Monte Carlo. A number of clusters K going from 1 to the number of sampling sites were tested. Simulations were run several times for each value of K. The most probable number of clusters was then estimated using the log-likelihood of the simulations or the Delta K, another estimator proposed by Evanno et al. (2005). The assignations of the individuals were averaged between the different simulations for each value of K.

Finally, an approximate Bayesian computation method implemented in DIYABC 2.0.3 (Cornuet *et al.* 2008) was used to compare introduction scenarios of Red-whiskered bulbuls on Reunion and to see if several introductions could explain the phenotypic differences observed between two populations. With this method a large number of datasets simulated according to invasion scenarios given by the user are compared. For each scenario, datasets are simulated using different combinations of parameters drawn in distributions set *a priori* by the user. Summary statistics are used to describe the simulated and real data sets. When all the datasets are simulated, only a defined fraction of them is kept (the datasets which are closer to the real one). The most probable scenario can then be identified by comparing the proportions of each scenario represented in the final group of datasets. The posterior distributions of the parameters can also be estimated by looking at the parameters values of the simulated datasets that are closest to the real data set.

III. Results

In this section, the results of the different parts of my thesis are presented in the form of three manuscripts. As an introduction to these manuscripts, I briefly summarize here their main findings.

III.1. Synthesis of the results

For both species, the phylogeographic analyses showed that the introduced populations I studied have the same source: bulbuls of Reunion, Mauritius and Oahu were found to come from the lowlands of Eastern India, and parakeets of Paris, Barcelona and Heidelberg were found to come from Asia. I concluded that phylogenetic origin was not a probable cause of phenotypic differences between introduced populations.

Classical and geometric morphometric approaches generally gave similar results, and the results obtained for bulbuls and parakeets were comparable. Indeed, in both cases, the morphology of the individuals belonging to introduced populations was significantly different than the morphology of those belonging to their source populations, showing a morphological differentiation since their introduction. Moreover, we also found significant morphological differences between individuals belonging to populations established in different environmental conditions. Possible explanations for this pattern are rapid adaptation to local conditions but also phenotypic plasticity and stochastic evolution resulting from recent demographic processes. The comparison of populations introduced in 'replicated' situations suggested that these differences were unlikely to be adaptive in both species.

The population genetics approach supported these results except in one case. Indeed, the study of microsatellite loci showed genetic differences between introduced populations indicating that recent demographic processes have caused a stochastic evolution in these populations. Moreover, the morphological differences found between populations corresponded to the genetic differences observed with neutral loci. This suggested that the cause of the morphological and genetic differentiations observed is the same. Stochastic evolution caused by recent demographic processes was therefore a more probable explanation to the phenotypic differences observed than rapid adaptation.

We however identified one case in which rapid adaptation has possibly occurred: for Red-whiskered bulbuls in Mauritius. Indeed, on this island, we found morphological differences between the two contrasted environments in the absence of genetic structure or differences in phylogenetic origin.

III.2. Organisation of the results in the manuscripts

The results synthesized here are detailed in the three following manuscripts. The first manuscript is about the population genetics of introduced Red-whiskered bulbuls. The second deals with the phylogeography and the morphology of Red-whiskered bulbuls. And finally, the phylogeography, morphology and population genetics of Ring-necked parakeets are gathered in the third manuscript.

Manuscript 1

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Rapid phenotypic changes in introduced populations of Red-whiskered bulbuls may result from stochastic evolution, rather than from rapid adaptation

Ariane Le Gros[1, 2, 3*], Philippe Clergeau[2], Sarah Samadi[3]

[1]Université Paris Diderot, Sorbonne Paris Cité, 75013 Paris, France

[2]Muséum National d'Histoire Naturelle, Département Ecologie et Gestion de la Biodiversité, UMR 7204 CESCO, 75005 Paris, France

[3]Muséum National d'Histoire Naturelle, Département Systématique et Evolution, UMR 7205 ISYEB, 75005 Paris, France

*Corresponding author: Ariane Le Gros, alegros@mnhn.fr

Abstract

In studies on biological invasions or global changes, correlations between rapid phenotypic changes and environmental modifications are often interpreted as the result of rapid adaptation. However, phenotypic plasticity and stochastic evolution can also induce phenotypic changes and have been little considered. Here we propose a comparative approach to facilitate the study of the mechanisms underlying rapid phenotypic changes. We used this approach on the case of the Red-whiskered bulbul (*Pycnonotus jocosus*). This invasive bird presents different morphotypes in places where it has been introduced and that differ in environmental conditions. This was interpreted as the result of rapid adaptation. We took advantage of the fact that this bird has been introduced in several islands presenting these different environments to compare the genetic structure of populations between replicates and assess the possible role of stochastic evolution on these phenotypic differences. Thanks to an approximate Bayesian computation method we reconstructed the invasion and demographic history of the bulbul in different introduced islands. We show that founder effect correlates with the observed morphological differences. This study thus emphasizes that rapid phenotypic changes, even if correlated to changes in the environments, may be driven by stochastic demographic processes rather than by natural selection.

Key words:

Rapid adaptation, evolution, stochasticity, invasive species, Approximate Bayesian Computation, *Pycnonotus jocosus.*

1. Introduction

Recently, there has been a growing interest in the scientific community for the study of evolution observed on ecological time-scales [1]. Among the reasons for studying such rapid and contemporary, evolutionary changes, improvement of biodiversity conservation and control of invasive species are frequently cited [1]. Indeed, anthropic activities are causing very rapid modifications of the environmental conditions and have the potential to impact strongly ecosystems [2]. This brought conservation biologists to wonder whether species impacted by rapid environmental changes such as global warming are able to adapt fast enough to avoid extinction [3]. In parallel, many introduced species have impacts on human health, biodiversity and world economy [4,5]. Identifying factors favouring biological invasions has therefore been, and is still, the goal of many studies on invasive species. Rapid adaptation has only recently been put forward as one of the processes that can favour the establishment and subsequent spread of introduced species [6,7].

Among the increasing number of studies pointing at such processes of rapid adaptation, many show that changes in morphology, life history, physiology and behaviour can be observed in only a few generations in various taxa [8]. Focusing on morphological changes in vertebrates, a few articles show striking cases of rapid changes in association with severe changes of environmental conditions. Phillips *et al.* [9] showed that the annual rate of progress of the cane toad invasion front increased fivefold within the 70 years following their introduction in Australia. This time-period corresponds to approximately 50 generations of cane toads. This increase in speed was correlated with an increase of the leg length of cane toads on the invasion front. Phillips *et al.* [9] hypothesized that spatial selection was triggering a rapid adaptive change in the front population which increased the dispersion abilities of cane toads. Similarly, a decrease in the wing length of cliff swallows was identified in 30 years in a population exposed to increasing road traffic [10]. The authors hypothesized that the diminution of wing length increased the flight precision of the swallows and that this evolution was a local adaptation resulting from the selective pressure created by road traffic. Losos *et al.* [11] experimentally introduced lizards to islands differing in vegetation height. Twelve years later, the morphology of the lizards had changed between the islands, notably in hindlimb length. These differences were correlated to the diameter of perches available for lizards on the different islands. The authors thus hypothesized that these morphological changes were a local adaptation to the islands vegetation.

In all these studies, the correlation between morphological changes and a rapid change of the environment was interpreted as the result of a rapid adaptation. However, two

mechanisms can induce such phenotypic changes: evolution (i.e. change in frequency of genotypes) and phenotypic plasticity. Moreover, evolution can be either adaptive (*i.e.* caused by selection) or non-adaptive (*i.e.* caused by stochastic demographic processes such as genetic drift and founder effect). To determine if a phenotypic change results from adaptive evolution, both its heritability and an associated increase in relative fitness need to be ascertained. Yet, as underlined by Merilä and Hendry [12], experimentally demonstrating adaptive evolution in the wild still represents a major challenge. We propose to use the fact that invasive species are often introduced independently in several places to develop a comparative approach for analysing the causes of rapid morphological evolution. Populations introduced from a same source in similar environments and during a similar length of time could be used as replicates of a pseudo-experiment on rapid adaptation. If all the replicates changed in the same way, we considered it was not by chance, whereas if they changed in different way, we considered it was caused by a stochastic process.

Here, we used this approach to investigate the possible causes of a phenotypic changes in Red-whiskered bulbuls (*Pycnonotus jocosus*). A previous study on this invasive bird had identified morphological differences between individuals recently introduced in an island correlating with two different environments within the island [15]. Rapid adaptation to local conditions was suggested to be the cause of these morphological differences. In order to assess the potential role of stochastic demographic processes, we took advantage of the fact that the Red-whiskered bulbul has been introduced in several islands presenting these two kinds of environments to compare the genetic structure of populations and their demographic history between replicated environments.

2. Methods

(a) Study species

The Red-whiskered bulbul, is an invasive passerine bird, native from South-East Asia. Its natural range includes the Indian sub-continent, Nepal, Southern China and Indochina [13]. This species is a popular cage bird and has been introduced in many regions of the Pacific and Indian oceans mainly during the second half of the 20th century [14].

Amiot *et al.* [15], showed on Reunion Island that the populations of Red-whiskered bulbul have a distinct beak morphology on the windward and leeward coasts, which present very contrasted environmental conditions. The only known introduction of Red-whiskered bulbuls took place from Mauritius in 1972 at the south-eastern point of the island [14,16]. The population rapidly expended from there, first on the humid and forested windward coast

during the 1980's and then on the drier leeward coast during the 1990's [16]. The two coasts are separated by a high mountain range which is likely to constitute an obstacle to the Red-whiskered bulbul dispersion and thus to isolate the populations from the two coasts [15]. As only one introduction event was reported the observed morphological differences between the two coasts were attributed to a local adaptation occurring in only 10 to 15 generations [15]. However, some Red-whiskered bulbuls were observed at the north-western point of Reunion (Sainte Marie) in 1978, well before the population could have reached this point by natural colonization from St Philippe [16].

The Red-whiskered bulbul was introduced in Mauritius and Oahu (Hawaiian archipelago) that also have a humid windward coast and a drier leeward coast. Historical sources suggest that the Red-whiskered bulbul was introduced on Mauritius, probably from India, in 1892. From there it was introduced on Reunion in 1972. Oahu was colonized at the same period as Reunion (1965) but from an unknown source [14]. The three islands have about the same size. On Oahu, like on Reunion, the windward and leeward coasts are separated by a high mountain range which is supposed to be an obstacle to the Red-whiskered bulbul dispersion whereas on Mauritius, the relief is lower and there are therefore no apparent geographic barriers to the bulbul dispersion.

(b) Samples

Feathers or toe-pads were obtained from 480 Red-whiskered bulbuls captured on Reunion, Mauritius and Oahu. On Reunion, 437 Red-whiskered bulbuls were captured during a control program organized by FDGDON (Fédération Départementale des Groupements de Défense contre les Organismes Nuisibles de la Réunion) in 2002 and 2003 [15]. They were captured in 12 sites spread along the windward and the leeward coasts (figure S1A). Toe-pads were collected on birds and stored in absolute ethanol at -20°C. In 2013, 50 Red-whiskered bulbuls were captured on Mauritius and 45 on Oahu with mist-nets. A few feathers were collected on each individual. The birds were then released and the feathers were stored dried at -20°C. On Mauritius, bulbuls were caught in three biological stations spread in the island and administrated by an NGO, the Mauritian Wildlife Foundation (figure S1B). On Oahu, the bulbuls were caught in two sites, one on the leeward coast and the other on the windward coast (figure S1C).

(c) DNA extraction and genotyping

DNA was isolated either from toe-pads or from the basal part of feathers. Total genomic DNA was extracted with a robot (Eppendorf epMotion 5075) and the Machery-Nagel NucleoSpin96Tissue kit following the manufacturer instructions. Dithiothreitol **(**DTT) was added to the digestion mix in order to facilitate keratin hydrolysis. Ten polymorphic microsatellite loci were selected from the literature (table S1). They were amplified in two separate multiplex and tagged with fluorescent forward primers (dyes: 6-FAM, VIC, NED, PET; Applied Biosystems). PCR amplifications were done using the following reagent quantities: 1.25µL of the primer mix (1µM of each primer and TE buffer), 4µL of RNase-free water (Qiagen), 6.25µL of 2x Type-it Multiplex PCR Master Mix (Qiagen) in a final volume of 11.5µL. The following cycling conditions were used: 95°C, 5 min.; (95°C, 30 sec.; 57°C, 90 sec.; 72°C, 30sec.) x 25 cycles; 60°C, 30 min. Samples were genotyped on an Applied Biosystems 3130XL DNA sequencer. Genotypes were scored with GeneMapper 4.0 (Applied Biosystems) and checked manually.

(d) Genetic diversity

The presence of null alleles was assessed with FREENA [17]. This program uses the expectation Maximization algorithm of Dempster to estimate null allele frequency. Sample sites with less than 20 individuals were excluded of the analysis. Mean number of alleles, Shannon's information index, observed heterozygosity, expected heterozygosity, unbiased expected heterozygosity, and fixation Index were assessed over all loci and for each sample site with GENEALEX 6.5 [18]. Deviation from Hardy-Weinberg equilibrium and linkage disequilibrium between pairs of loci were also tested for each sample site with GENEPOP 4.2.1 [19] using default parameter values.

(e) Genetic structure

The Bayesian clustering approach implemented in STRUCTURE 2.3.3 [20,21] was used to describe the genetic structure in the data set. Twenty five runs were performed for each value of K from 1 to 17 (burn-in period: 25.10^3, 75.10^3 iterations). The admixture model and the assumption of correlated allele frequencies were chosen. The sampling location were used as prior information to assist clustering thanks to the "LOCPRIOR" option. According to Hubisz *et al.* [22] this model does not find non-existing genetic structure and is able to ignore the sampling information when necessary. The most likely number of clusters (K) was inferred by

looking at the variation of the likelihood of the data. Convergence of the MCMC was assessed by checking the stabilization of the parameters α and F.

As the sampling in Reunion is well spread along the coasts and also more extensive than in the other islands, the Bayesian clustering software GENELAND 3.1.5 [23–25] was used to study more precisely the genetic structure within Reunion Island. Contrary to STRUCTURE, GENELAND takes into account the geographical coordinates of the samples. GENELAND also allows using loci displaying null alleles. The model with uncorrelated allele frequencies was used as recommended when some loci have null alleles. The parameters were set as following: number of iterations = 5.10^6, sampling frequency = 100, burnin = 1.10^4, maximum rate of Poisson process = 387, uncertainty on coordinates = 0.1, and maximum number of nuclei in the Poisson-Voronoi tessellation =1.10^3. Values between one and six were tested for the number of groups in ten runs. GENELAND automatically selects the most probable number of group for each run. The run with the highest mean posterior density was kept. The MCMC convergence was assessed by checking the trace stabilization.

(f) Isolation by distance (IBD) and migration on Reunion

The natural logarithm of the linearized genetic differentiation between each sampling site: Fst/ (1-Fst), was used as genetic distance to test for IBD. Fst values were corrected for the possible presence of null alleles with FREENA. Significance of the Fst values was tested using 10,000 permutations. Two kinds of geographic distances were used. First, the mountain range was not considered as an obstacle and the bird eye distance was used as geographic distance (Euclidian distance). Secondly, the mountain range was considered as an obstacle and the distances between each sampling site were calculated following the coastline. In both cases the distances were log-transformed.

IBD between sampling sites was tested first on the whole island, and then within the groups delimitated by STRUCTURE and GENELAND. This approach allows to account for the effect of "by chance" geographic separation of groups that are also very differentiated genetically [26]. Indeed, if some groups are very differentiated genetically and also isolated in space by chance, the comparisons between sampling sites on the whole island will artificially increase the IBD pattern. The P-values of the correlation coefficients were computed using a Mantel test (1000 permutations).

Actual migration rates between the previously defined groups were assessed with BAYESASS 1.3 [27]. The parameters were chosen to fit with the author's recommendations: mixing parameters for allele frequencies, inbreeding coefficients and migration rates were set

to 0.12, 0.15 and 0.07 respectively, number of iterations = 1.10^7, burnin = 1.10^6, and sampling frequency = 100. The MCMC convergence was assessed by checking the trace stabilization.

(g) Comparison of invasion scenarios

An approximate Bayesian computation method implemented in DIYABC 2.0.3 [28] was used to assess whether the two groups delimitated by STRUCTURE and GENELAND on Reunion were founded by the same or different sources. The origin of the bulbuls on Oahu was also investigated to check if it was or not similar to the source of Mauritius and Reunion. . With this method a large number of datasets simulated according to invasion scenarios given by the users are compared. For each scenario, datasets are simulated using different combinations of parameters drawn in distributions set *a priori* by the user. Summary statistics are used to describe the simulated and real data sets. When all the datasets are simulated, only those that are closer to the real one are kept. The most probable scenario can then be identified by comparing the proportions of each scenario represented in the final group of datasets. The posterior distributions of the parameters can also be estimated by looking at the parameters values of the simulated datasets that are closest to the real data set.

Two families of scenarios were compared. In the first family, the population of Oahu was founded by individuals related to the populations in Mauritius and Reunion (figure S2, scenarios A, B, C and D). In the second family, the population of Oahu was founded by a different source (figure S2, scenarios E, F, G and H). Within these families, scenarios on Reunion colonization were either with two independent introductions forming the two populations identified with morphological data (figure S2, scenarios A, B, E and F) or with only one introduction and then a split of the founding population (figure S2, scenarios C, D, G and H). According to historical data Mauritius was used in both cases as the source population of the introductions on Reunion. Eight scenarios representing all the possible combinations were tested. The demographic parameters were set as realistically as possible (Table S2). The microsatellite loci were separated in three groups according to their repeat motif. The mutation model parameters were left to default values. The chosen within sample summary statistics were the means of number of alleles, genetic diversity, size variance and Garza-Williamson's M. For among samples summary statistics, we used the means of the number of alleles, genetic diversity, size variance and the Fst values, shared allele distances and $(d\mu)^2$ distances . For each scenario 750 000 data sets were simulated and the posterior probability was assessed with the regression method using the 0.1% closest simulated data sets. In order

to gain more statistical power for the study of the introduction in Reunion, only the most probable family of scenarios explaining the introduction in Oahu was kept. For each of the four remaining scenarios 1.5 million data sets were simulated and their posterior probabilities were assessed using the same method as before. Type I errors (probability to reject the scenario although it is true) and type II errors (probability to accept the scenario whereas it is false) were calculated for the most probable family of scenarios with the logistic regression approach on 6000 simulated datasets, using the same parameters distributions and mutation models as before. The "linear discriminant analysis on Summary Statistics" option was used to reduce the computation time. The sample sites that seemed admixed were not used in these analyses.

3. Results

(a) Genetic diversity

Amplification of the microsatellite loci was successful with only 1.8% of missing data over all loci and individuals. A null allele was detected at locus TG05-046 with FREENA. The null allele frequency at this locus was over 10% in 55% of the tested sample sites. Therefore, in the following analyses, a dataset corrected by FREENA for the presence of null alleles was used when possible. Otherwise, the data from locus TG05-046 were discarded.

As expected in invasive populations the genetic diversity was not very high (table 3).Overall, sample sites on Mauritius were slightly more diverse than the ones sampled in Reunion or Oahu. None of the sample sites significantly deviated from Hardy-Weinberg equilibrium nor presented linkage disequilibrium (table S3).

(b) Genetic structure

The log likelihood of the simulations run with STRUCTURE increased sharply until K=3, and started decreasing slowly from K=4 (figure S3). For these values of K, the 25 runs gave the similar results. For K=3, all individuals from Oahu were assigned in a single cluster. The individuals from Reunion were separated into two clusters: the sites W3, W4 and W5 (blue cluster) and the other sites (orange cluster). The admixture was low (inferior to 33%) in all sampled sites except in the sites W2 and L1. All the individuals of site W2 had a mixed origin attributed to the blue cluster for 55% in average and to the orange cluster for 45% in average. In the site L1 most of the individuals had a blue origin but 27% of the individuals had more than 50% of their origin attributed to the orange cluster. The limit of the clusters did not exactly match the separation between the windward and leeward coasts. However, the sample

sites were sorted in the same two main groups as those defined by Amiot *et al.* [15] and based on the beak morphology of male bulbuls. Individuals from Mauritius were divided in equal part into the two clusters identified on Reunion. For K=4, all individuals from Mauritius were grouped in a single cluster and were well separated from the others although some individuals were partially assigned to the blue cluster of Reunion. The three clusters identified previously on Oahu and Reunion remained the same. The site W2 was still in between the orange and blue clusters. Starting from K=5, no supplementary clusters were defined (figure 1).

Within Reunion Island, the analysis with GENELAND showed that the most likely number of clusters was two. The sample sites were grouped as in the STRUCTURE analysis (figure 2). This analysis also showed that the two clusters were well separated as the probability of belonging to one cluster dropped rapidly at the frontier between the two clusters. The sites W2 and L1 were located on this limit.

(c) Isolation by distance and migration on Reunion

When all sampled sites were considered, the correlation between genetic and geographic distances, whatever the chosen distance, was significant and positive. Geographic distances calculated following the coastlines explained better the genetic differences between sample sites than Euclidian distances (r=0.45, P=0.004 versus r=0.41, P=0,001). However, within each cluster, the correlation between genetic and geographic distances, was not significant (orange cluster: Euclidian distances P=0.32, coastline distances P=0.34; blue cluster: Euclidian distances P=0.36, coastline distances P=0.33). Actual migration rates of 4% per generation from the orange to the blue cluster and 5% in the other way were estimated with BAYESASS.

(d) Comparison of invasion scenarios

The scenarios in which the bulbuls from Oahu derive from the same source as those from Mauritius and Reunion had the highest posterior probability (mean posterior probability of scenario A, B, C and D: 0.21 against 0.044 for scenarios E, F, G and H). These four scenarios were then compared using more simulated data sets. The scenario with two independent introductions on Reunion had the highest posterior probability. In this scenario, a first introduction from Mauritius formed the blue cluster and a second introduction also from Mauritius formed the orange cluster later on (posterior probability of scenario A: 0.51 against 0.16 for scenario B, 0.23 for scenario C and 0.081 for scenario D; figure 3). The 95% confidence interval showed that scenario A can be confidently discriminated from the three

other ones (figure 3). However, Type I and type II errors of all our scenarios were around 40% (Type I error for scenario A: 0.44, B: 0.46, C: 0.37 and D: 0.40; Type II error for scenario A: 0.46, B: 0.43, C: 0.40 and D: 0.373). This was mainly caused by the fact that the scenarios A and B and the scenarios C and D were too similar to be well discriminated. The type I and type II errors dropped to about 20% when the scenarios of a couple were considered together (Type I error for scenario A & B: 0.21 and C & D: 0.19; Type II error for scenario A & B: 0.19 and C & D: 0.20).

4. Discussion

(a) A well-defined genetic structure between islands and within Reunion

The analysis of our genetic data set shows a differentiation of the populations between the three islands but also within Reunion Island. Individuals from Oahu form a distinct genetic group. The hypothesis that Red-whiskered bulbuls from Mauritius are the source of the introduction on Reunion was supported. Interestingly, the study sites on Reunion were divided between the two clusters in the same way with genetic and morphological data. It is therefore possible that the cause of the genetic structure we observe is also the cause of the morphological structured observed in by Amiot *et al.*[15]. Individuals caught at site W2, were assigned in almost equal proportions to the two clusters. This site is located at the geographic limit between the two clusters. This pattern can be explained by two hypotheses: either there was a progressive differentiation of the individuals in the blue cluster as they colonized the coast towards the North, or the two clusters correspond to independently founded populations and, as their territory expended, a contact zone was formed between them.

The results obtained with GENELAND tend to confirm the second hypothesis as the probability of cluster membership presents a sharp shift between the two clusters instead of a gradual transition. The fact that no geographical structure was found on Mauritius and Oahu also supports this hypothesis. Indeed, both islands have the same size as Reunion and the relief and time since the Red-whiskered bulbul introduction is similar in Reunion and Oahu. It therefore seems that these islands are either too small and their relief is not creating a sufficient barrier to dispersion, and/or there was not enough time for populations to differentiate in-situ after the colonization of the islands.

(b) No evidence for isolation by distance on Reunion

Isolation by distance is detected when sampling sites from the whole island are compared but not within clusters. This observed pattern is probably generated by the strong genetic

differences existing between the two clusters and their distinct geographic position. Moreover, we estimated with BAYESASS that the actual migration rate between the two clusters reached about 5% per generation. This indicates that they are some gene flows between the clusters. Thus, we conclude that isolation is not the cause of the genetic structure we observe on Reunion. This is supported by the lack of genetic differentiation on Oahu whereas it presents the same geographical characteristics as Reunion Island.

(c) An unexpected invasion history

The comparison of invasion scenarios with DIYABC shows that the populations on the three islands were probably founded by the same source. Contrary to historical data, the Red-whiskered bulbul was probably introduced twice on Reunion, once in the South and once in the North. In the scenario with the highest posterior probability, the blue cluster was founded first which would explain why it is more represented on the island, and the orange cluster was founded later on.

(d) Founder effect as the cause of the observed morphological differentiation

The genetic structure of the Red-whiskered bulbul on Reunion, coincides with the morphological differentiation observed by Amiot *et al.* [15]. However, within each population (and thus morphotype) there was no isolation by distance on Reunion. Moreover, the comparison of the genetic structure on Reunion with that of Mauritius and Oahu showed that isolation and drift alone cannot explain the pattern we observe on Reunion. Indeed, there was no evidence for genetic structure on Mauritius and Oahu although similar natural barrier are present. Thanks to the comparison of invasion scenarios, we inferred that the populations of Red-whiskered bulbul on Reunion were founded by two introduction events. We conclude that a different origin, rather than local adaptation, is probably the cause of the genetic and morphological differences observed between the two coasts of Reunion. This is supported by the findings of Roussel *et al.* [29] who showed with an isotopic analysis that they were no differences in diet or feeding behaviour between bulbuls of the two coasts of Reunion. In that case, several non-exclusive mechanisms can explain the genetic and morphological differences between the two populations: 1) because of a sampling effect, the individuals which founded the two populations already had different genetic backgrounds and morphologies at the moment of the introduction, 2) drift and/or selection created the observed differences in-situ after the introduction.

Although founder effects are a likely explanation for the genetic structure we observe, rapid local adaptation and phenotypic plasticity cannot be totally excluded as possible causes for the morphological structured observed previously. Complementary approaches such as common garden experiments, reciprocal transplants, the study of pedigrees, or the study of evolution in genes associated with morphology, would give some further elements to specify the role of these alternative mechanisms [12]. A more practicable approach could be the comparative study of the phenotypes of Red-whiskered bulbuls in Reunion, Mauritius, Oahu and in their native range.

(e) Stochastic evolution: an under estimated cause of rapid phenotypic changes

During biological invasions, several mechanisms can modify the standing genetic diversity of a population and thus affect its evolutionary potential [30]. Founder effect, because of the random sampling of individuals in the source population can decrease the genetic diversity the population at its introduction. If the founder population remains small for some time, genetic drift will further decrease genetic diversity with the random loss of some alleles. On the contrary, multiple introductions have recently been suggested as a mechanism increasing the genetic diversity in invasive populations [31–33]. Admixture between introduced populations with different genetic background can indeed partly restore the genetic diversity found in the native area.

Thus, stochastic evolution cannot be excluded as a potential mechanism for the observed phenotypic change in studies on rapid adaption. For example, as cane toads were introduced in Australia for biological control, they are likely to have been introduced several times and in different places. The populations at the invasion front could then result from a mix of different populations. This could explain the morphological differentiation observed by Phillips *et al.* [9]. Similarly, the number of lizards introduced on each island in the study of Losos *et al.* [11] was probably small. Founder effect and genetic drift are therefore possible causes for the morphological differentiation observed between islands. In this article, we show an example of morphological differentiation that was interpreted as the result of rapid adaptation and which was in fact probably caused by founder effects. We thus highlight the importance of taking stochastic evolution into accounts in studies on rapid evolution and suggest a comparative approach to facilitate the study of mechanisms causing rapid phenotypic changes.

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Figures

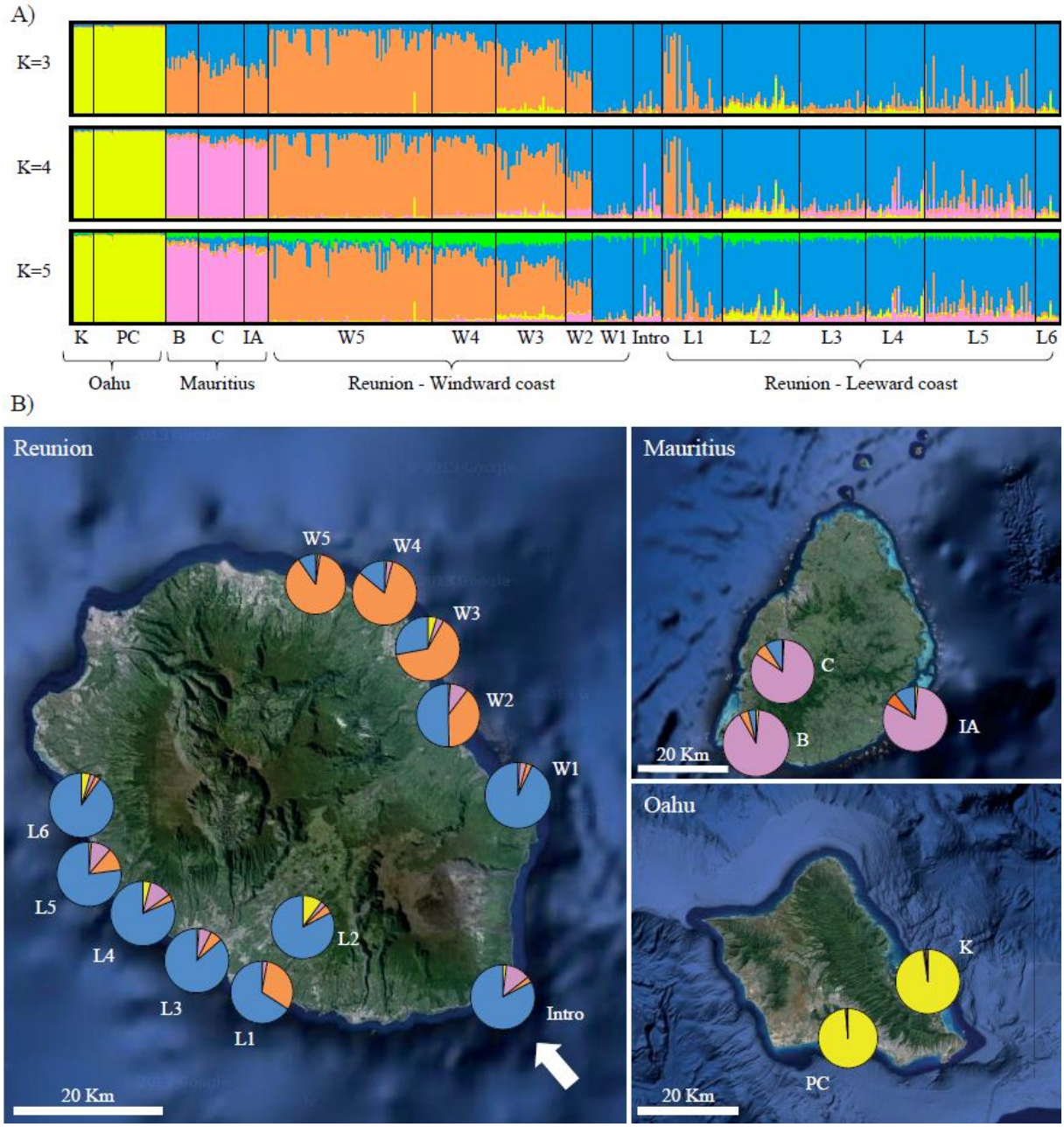

Fig. 1: A) Cluster assignments of individuals obtained with STRUCTURE for K=3, 4 and 5. The results of the 25 runs were pooled together using Clumpp [34]. Each vertical line represents a single individual and individuals are grouped by sampling site and island. B) Average cluster assignments of individuals for each sampling site in the case where K= 4. The white arrow indicates the location of the introduction of Red-whiskered bulbuls on Reunion in 1972.

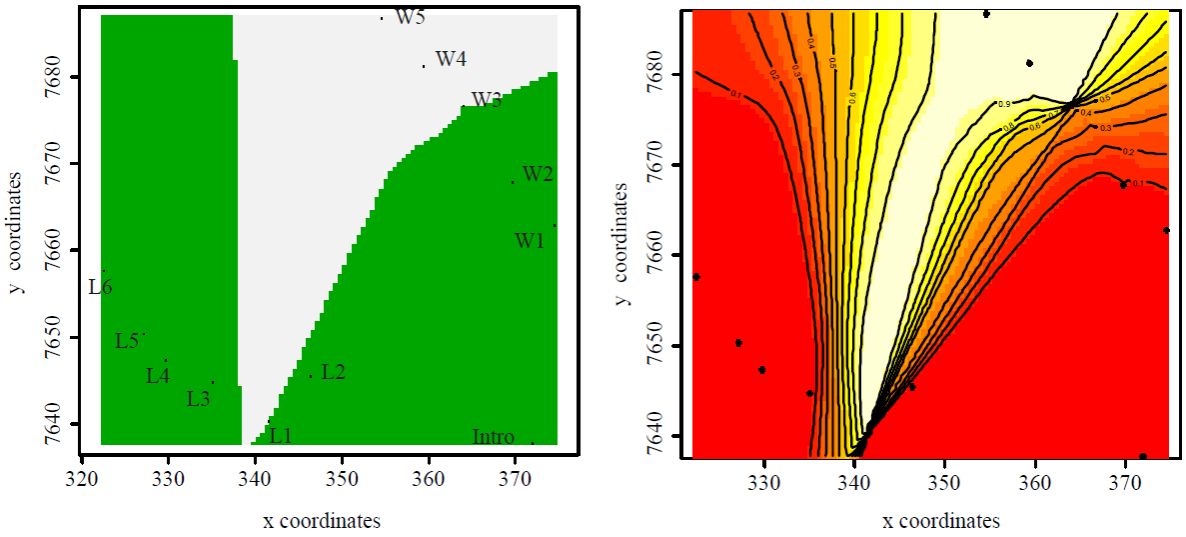

Fig. 2: Maps obtained with GENELAND. Left: clusters delimitation. Right: curves indicating the probability to belong to the white cluster. Black dots represent sampling sites. Red indicates a low probability of belonging to the white cluster and light-yellow, a high probability of belonging to the white cluster.

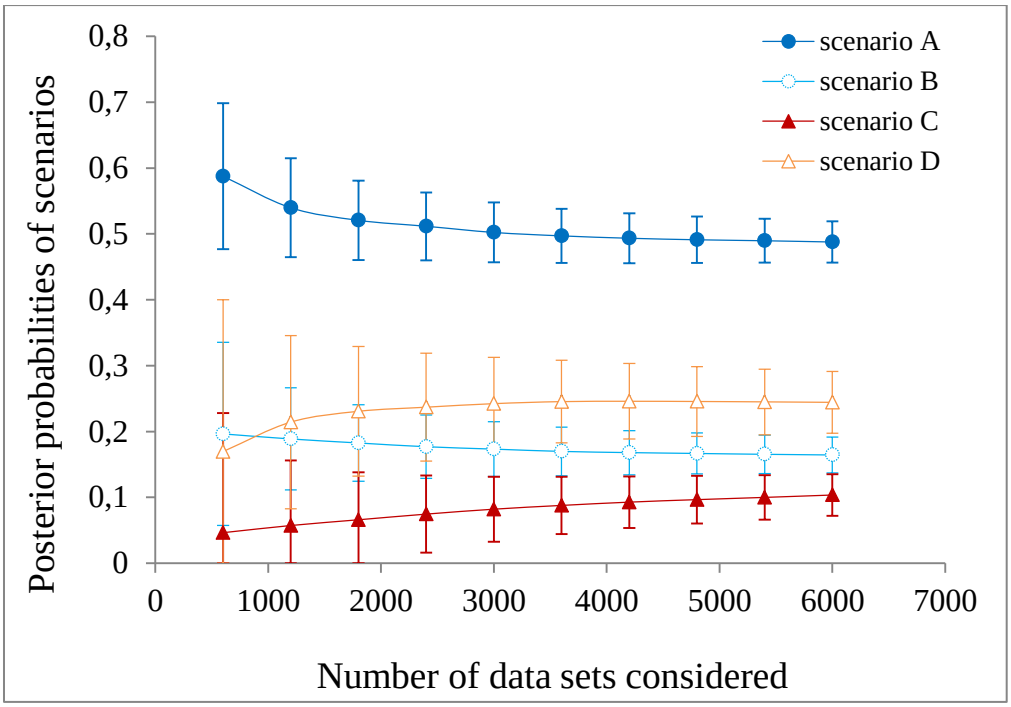

Fig. 3: Posterior probabilities of scenarios A, B, C and D calculated with the logistic regression approach implemented in DIYABC on 6000 simulated data sets. The error bars represent the 95% confidence interval calculated with DIYABC.

Supplementary Material

1) Tables

Table S1: Description of the microsatellite markers used in the study. Na: number of alleles.

locus	Na	primers	motif	label	source
Pca3	4	F:GGTGTTTGTGAGCCGGGG R:TGTTACAACCAAAGCGGTCATTTG	$(GT)_6CT(GT)_3$	6-Fam	Dawson et al. (2000)
TG04-004	2	F:CTGGAGCAGTATTTATATTGATCTTCC R:GAAGATGTGTTTCACAGCATAACTG	$(AT)_{10}GT(AT)_7$	Pet	Dawson et al. (2010)
Pfl35	6	F:GTGCAGTTTCGGTTGTTTCCC R:CCATGGTACTGTTAGAGATCGGTATC	$(TAGA)_8$	Ned	Lokugalappatti et al. (2008)
TG13-009	3	F:TGTGGTGGGATAGTGGACTG R:CTGTAAAATGTGCAAGTAACAGAGC	$(AT)_4GT(AT)_5$	Vic	Dawson et al. (2010)
TG05-053	4	F:GCATCATCTGGTTGAACTCTC R:ACCCTGTTTACAGTGAGGTGTT	$(T)_4GA(T)_6AA(T)_{16}AA(T)_4G(T)_6$	6-Fam	Dawson et al. (2010)
TG01-040	3	F:TGGCAATGGTGAGAAGTTTG R:AGAATTTGTACAGAGGTAATGCACTG	$(AT)_2G(AT)_7AC(AT)_6$ $TT(AT)_2$	Pet	Dawson et al. (2010)
TG05-046	5	F:AAAACATGGCTTACAAACTGG R:GCTCAGATAAGGGAGAAAACAG	$(AT)_8$ $(A)_4$ $(AT)_6$ $(A)_9$ $(AT)_2$	6-Fam	Dawson et al. (2010)
Ase19	3	F:TAGGGTCCCAGGGAGGAAG R:TCTGCCCATTAGGGAAAAGTC	$(CA)_4GA(CA)_5$	6-Fam	Richardson et al. (2000)
Ase18	8	F:ATCCAGTCTTCGCAAAAGCC R:TGCCCCAGAGGGAAGAAG	$(GT)_{12}$	Ned	Richardson et al. (2000)
Ase55	2	F:GTGTGGACTCTGGTGGCTC R:TCCCAAAGCACTCAAACTAGG	$(GT)_9$	Pet	Richardson et al. (2000)

Table S2: Prior distributions of the historical parameters used in the introduction scenarios modelled with DIYABC. N_x: number of individuals in the population x (constant in time), N_{bx}: number of individuals in the population x during the bottleneck following introduction (constant during the whole bottleneck), t_i: number of generations between present and an introduction event or a split i, t_{bi}: number of generations during the bottleneck following an introduction event i.

	distribution	min	max
N_M	uniform	100	10000
N_H	uniform	100	10000
N_{RB}	uniform	100	10000
N_{RO}	uniform	100	10000
N_{S1}	uniform	100	10000
N_{S2}	uniform	100	10000
N_{b1}	uniform	2	100
N_{b2}	uniform	2	100
N_{b3}	uniform	2	100
N_{b4}	uniform	2	100
t_0	uniform	150	1000
t_1	uniform	50	150
t_2	uniform	20	55
t_3	uniform	10	35
t_4	uniform	5	25
t_{b1}	uniform	0	5
t_{b2}	uniform	0	5
t_{b3}	uniform	0	5
t_{b4}	uniform	0	5

Table S3: Mean number of individuals (N), number of alleles (Na), Shannon's diversity Index (I), observed heterozygosity (Ho), expected heterozygosity (He), unbiased expected heterozygosity (uHe), fixation index (F) per sampling site and over all loci. Proportion of loci deviating from Hardy-Weinberg equilibrium (HWE) and proportion of pair of loci showing linkage disequilibrium (LD) per sampling site.

		N	Na	I	Ho	He	uHe	F	HWE	LD
Oahu	**H-K**	9.80 (+/-0.20)	2.10 (+/-0.28)	0.57 (+/-0.11)	0.36 (+/-0.09)	0.37 (+/-0.07)	0.39 (+/-0.07)	0.05 (+/-0.11)	0/8	3/28
	H-PC	34.6 (+/-0.27)	2.30 (+/-0.37)	0.59 (+/-0.14)	0.35 (+/-0.07)	0.36 (+/-0.07)	0.37 (+/-0.07)	0.02 (+/-0.07)	1/8	1/28
Mauritius	**M-B**	16.00 (+/-0.00)	3.20 (+/-0.39)	0.77 (+/-0.14)	0.40 (+/-0.08)	0.42 (+/-0.07)	0.43 (+/-0.08)	0.09 (+/-0.07)	1/10	0/45
	M-C	22.00 (+/-0.00)	3.30 (+/-0.45)	0.83 (+/-0.16)	0.52 (+/-0.10)	0.46 (+/-0.08)	0.47 (+/-0.08)	-0.07 (+/-0.10)	1/10	1/45
	M-IA	11.90 (+/-0.10)	2.80 (+/-0.42)	0.74 (+/-0.15)	0.48 (+/-0.09)	0.43 (+/-0.08)	0.45 (+/-0.08)	-0.09 (+/-0.06)	0/8	1/36
Reunion	**W5**	78.80 (+/-0.47)	3.40 (+/-0.37)	0.74 (+/-0.13)	0.37 (+/-0.07)	0.42 (+/-0.07)	0.42 (+/-0.07)	0.11 (+/-0.09)	3/10	4/45
	W4	30.60 (+/-0.27)	3.00 (+/-0.37)	0.70 (+/-0.14)	0.40 (+/-0.09)	0.36 (+/-0.08)	0.40 (+/-0.08)	0.07 (+/-0.12)	1/9	2/45
	W3	33.20 (+/-0.42)	3.10 (+/-0.35)	0.75 (+/-0.15)	0.44 (+/-0.09)	0.42 (+/-0.08)	0.43 (+/-0.08)	0.01 (+/-0.08)	2/10	2/45
	W2	12.90 (+/-0.10)	2.70 (+/-0.40)	0.74 (+/-0.16)	0.41 (+/-0.09)	0.43 (+/-0.08)	0.45 (+/-0.09)	0.04 (+/-0.08)	0/8	0/36
	W1	19.90 (+/-0.10)	3.20 (+/-0.36)	0.73 (+/-0.13)	0.40 (+/-0.08)	0.41 (+/-0.07)	0.42 (+/-0.07)	0.06 (+/-0.08)	3/9	3/45
	Intro	13.90 (+/-0.10)	2.90 (+/-0.46)	0.75 (+/-0.15)	0.45 (+/-0.09)	0.44 (+/-0.09)	0.45 (+/-0.08)	-0.02 (+/-0.09)	1/8	0/36
	L1	27.60 (+/-0.87)	2.80 (+/-0.42)	0.73 (+/-0.16)	0.41 (+/-0.08)	0.42 (+/-0.09)	0.43 (+/-0.09)	-0.01 (+/-0.07)	0/8	4/36
	L2	36.70 (+/-0.62)	2.90 (+/-0.50)	0.72 (+/-0.18)	0.41 (+/-0.10)	0.41 (+/-0.09)	0.41 (+/-0.09)	-0.03 (+/-0.05)	1/8	1/36
	L3	31.40 (+/-0.27)	3.30 (+/-0.40)	0.81 (+/-0.13)	0.44 (+/-0.09)	0.46 (+/-0.07)	0.47 (+/-0.07)	0.09 (+/-0.12)	3/10	6/45
	L4	28.40 (+/-0.22)	3.50 (+/-0.48)	0.80 (+/-0.15)	0.45 (+/-0.09)	0.44 (+/-0.08)	0.45 (+/-0.08)	0.05 (+/-0.09)	3/10	3/45
	L5	52.80 (+/-0.61)	3.60 (+/-0.50)	0.81 (+/-0.14)	0.42 (+/-0.08)	0.46 (+/-0.08)	0.46 (+/-0.08)	0.12 (+/-0.08)	3/10	4/45
	L6	10.80 (+/-0.20)	2.50 (+/-0.31)	0.67 (+/-0.14)	0.38 (+/-0.09)	0.40 (+/-0.08)	0.42 (+/-0.09)	0.02 (+/-0.11)	1/7	0/36

Table S4: Sample sites

Island	Site ID	Site Name
Reunion	W5	Sainte-Suzanne
	W4	Saint-André
	W3	Bras-Panon
	W2	Saint-Benoît
	W1	Sainte-Rose
	Intro	Saint-Philippe
	L1	Saint-Pierre
	L2	Le Tampon
	L3	Saint-Louis
	L4	Etang-Salé
	L5	Les Avirons
	L6	Saint-Leu
Mauritius	B	Bel Ombre field station
	C	Camp field station
	IA	Ile aux aigrettes
Oahu	K	Kanenohe

2) Figures

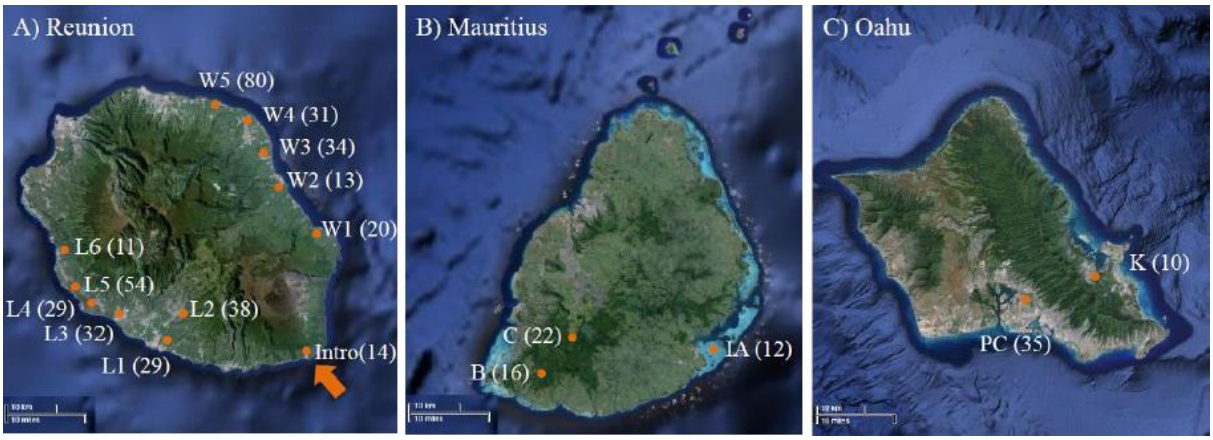

Fig. S1: Sampling sites on Reunion, Mauritius and Oahu (Hawaii). The number of individuals caught at each site is in brackets. The arrow shows the location on Reunion where the Red-whiskered bulbul was introduction in 1972.

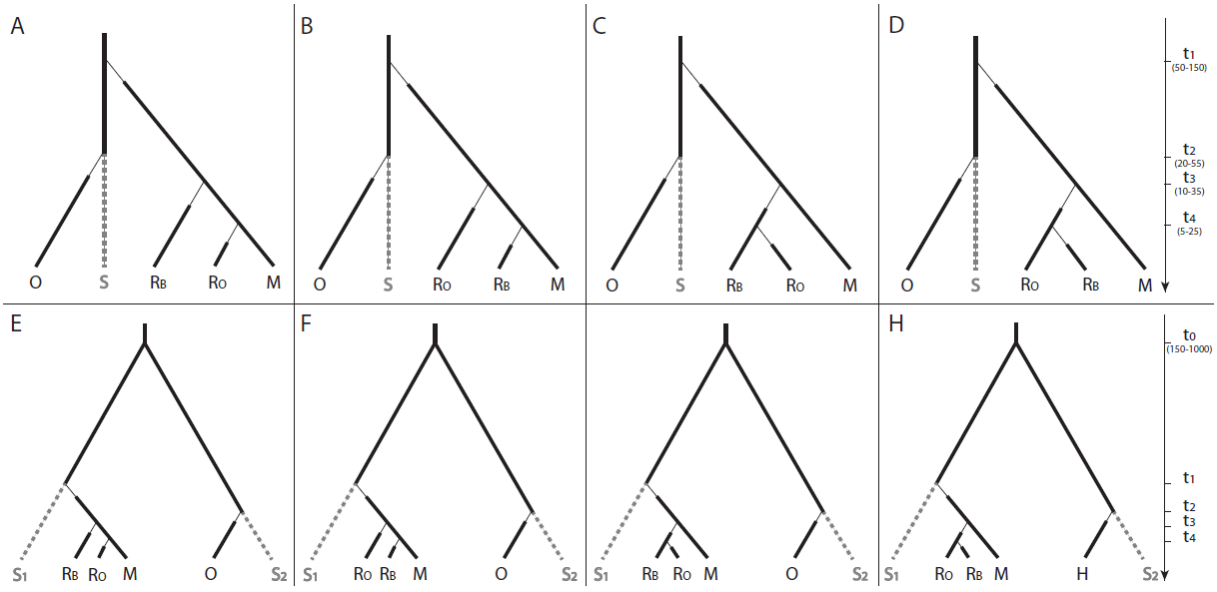

Fig. S2: Scenarii compared with DIYABC. Populations S: Source, S_1: Source 1, S_2: Source 2, M: Mauritius, O: Oahu, R_B: Reunion blue cluster, R_O: Reunion orange cluster. The large lines represent population of constant size. The thin lines represent the duration of bottlenecks. The dashed lines represent the trajectories of unsampled populations. The time scale is given on the right, t_0, t_1, t_2, t_3 and t_4 are the number of generations since a foundation event or a split.

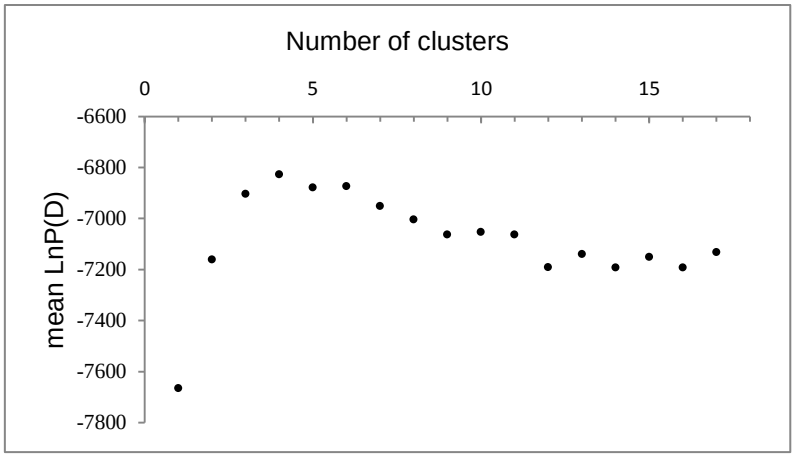

Fig. S3: Mean log likelihood of the simulations run with STRUCTURE over 25 runs and for each number of clusters.

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Manuscript 2

In preparation

Stochastic evolution explains phenotypic differences observed between populations of Red-whiskered bulbuls recently introduced in different environments

Ariane Le Gros[1, 2, 3*], Dario Zuccon[2], Raphaël Cornette[2], Blake Mathhys[4], Philippe Clergeau[3] and Sarah Samadi[2]

[1]Université Paris Diderot, Sorbonne Paris Cité, 75013 Paris, France
[2]Muséum National d'Histoire Naturelle, Département Systématique et Evolution, UMR 7205 ISYEB, 75005 Paris, France
[3]Muséum National d'Histoire Naturelle, Département Ecologie et Gestion de la Biodiversité, UMR 7204 CESCO, 75005 Paris, France
[4]Ohio Dominican University, Mathematics Computer and Natural Sciences Department, Columbus, OH 43219, United States
*Corresponding author: Ariane Le Gros, alegros@mnhn.fr

Abstract

Surprisingly, invasive species sometimes settle in environments that are very different from their native one. A number of studies on biological invasions report rapid phenotypic changes in populations introduced in new environments. It has often been hypothesized that these changes result from rapid adaptation, which would explain how invasive species can establish in new environments. However, because of the difficulties of experimentally demonstrating adaptive evolution, only a few recognized cases of rapid adaptation have been reported until now. Here we used a comparative approach to identify potential cases of rapid adaptation. We compared morphological traits between populations of a very successful invasive species, recently introduced in several kinds of environment: the Red-whiskered bulbuls (*Pycnonotus jocosus*). We then assessed whether historical or demographic factors could explain the morphological differentiations observed. All the introduced populations we studied were morphologically different from their phylogenetic source. Overall, the recent demographic history explained the morphological differentiations observed except in one case. This approach thus allowed identifying one case in which rapid adaptation might have occurred. However, in most cases, we show that the morphological differentiations between populations were caused by stochastic demographic processes. These results are in agreement with those of a population genetics study conducted previously on the same populations. We thus argue that when rapid phenotypic changes are observed in an introduced population, checking the

role of historical factors on these changes is a first step to take before concluding that they result from rapid adaptation.

Introduction

Among biological invasions, there are many examples of species that became invasive despite having been introduced in environments different from their native one. For example, the Ring-necked parakeet (*Psittacula krameri*), native of the Indian subcontinent and sub-Saharan Africa, has been widely traded as pet. Within a few decades, it has become established in North America, Europe and the Middle East (Lever 2010; Clergeau and Vergnes 2011). Similarly, the common carp (*Cyprinus carpio*), native of Eastern Europe and central Asia, was introduced deliberately for aquaculture and ornamental purpose. It is now invasive in Europe, Asia, Africa, North, Central and South America, Australia and Oceania (Lever 1996). In addition, introduced populations of this species were found to occupy a wide range of habitats varying in temperature, salinity, pH and oxygen levels (Koehn 2004).

Some studies on biological invasions report cases of rapid phenotypic changes (sometimes in less than 10 generations) in introduced populations, for example in plants (*e.g.* Maron et al. 2007; Montague et al. 2008; Kooyers and Olsen 2012) or insects (Huey et al. 2000; Gilchrist et al. 2004) introduced in environments with new climatic conditions. In vertebrates, cases of phenotypic changes following introduction in new environments were also reported, for example in fish introduced in lakes with new preys (Adachi et al. 2012), in birds and reptiles introduced in islands with different vegetation (Losos et al. 1997; Amiot et al. 2007), in amphibians and fish introduced in environment with new predators (O'Steen et al. 2002; Phillips and Shine 2005), and in mammals introduced in environment with new competition regimes (Yom-Tov et al. 1999). However, general mechanisms that enable species to establish in new environments have not yet been identified (Facon et al. 2006; Marsico et al. 2010).

Phenotypic plasticity, *i.e.* the ability of an organisms to express different phenotypes depending on biotic or abiotic environment (Agrawal 2001), has often been suggested to explain how some species can become invasive in such a wide range of environmental conditions (Claridge and Franklin 2002; Richards et al. 2006). In a recent meta-analysis the invasive species studied were found to express greater phenotypic plasticity than the non-invasive species. However, this plasticity was associated with a fitness benefit only in some cases (Davidson et al. 2011). These results emphasizes that phenotypic plasticity is probably not the only mechanisms favoring species establishment in new habitats.

Recently, there has been a growing interest for evolution happening on ecological time scales – i.e. contemporary evolution (Stockwell et al. 2003; Carroll et al. 2007). Indeed, theoretical and experimental studies have demonstrated that evolution has the potential to interact with ecological processes and thus impact population dynamics (Yoshida et al. 2003; Becks et al. 2010; Mougi 2012; Cortez and Weitz 2014). Several authors thus formulated the hypothesis that rapid adaptation is a factor favoring the establishment of invasive species in new environments (Sakai et al. 2001; Lee 2002; Lambrinos 2004; Prentis et al. 2008).

Following the emergence of this hypothesis, some studies tried to identify cases of rapid adaptation in populations recently introduced in new environments. However, even if a rapid phenotypic change is observed in a population, it can be either adaptive or results from stochastic evolution (*i.e.* changes in allele frequencies in a population because of stochastic demographic processes such as founder effects and bottlenecks). Moreover, if the phenotypic change is found to be adaptive, it can be caused either by phenotypic plasticity or by rapid adaptation. Therefore, in order to assess if a phenotypic change results from adaptation, both its associated increase in relative fitness and its heritability need to be ascertained. Yet, experimentally testing these two characteristics in the wild still represents a major challenge (Merilä and Hendry 2014). It is therefore still unsure if rapid adaptation plays a role in the establishment of invasive species.

Here we propose to use a comparative approach to identify possible cases of rapid adaptation in an invasive species. Indeed, invasive species are often introduced several times from the same source population, like for examples rats introduced to offshore islands from the mainland (Abdelkrim et al. 2010). Populations introduced in similar environments and at similar times can be considered as replicates of a pseudo-experimental set-up to assess whether rapid adaptation has occurred in these populations (Firmat et al. 2012). Like in an experiment, if similar phenotypic changes are observed in populations introduced in replicated environments, it is improbable that it happened by chance and is thus likely to be the result of selection. On the contrary, if the phenotypic changes are different between replicates, it is likely to be the result of stochastic processes. If potential cases of rapid adaptation are identified, the use of complementary approaches such as common garden experiments, can be considered to test whether the observed changes are adaptive and heritable.

We applied this comparative approach to the case of an invasive bird: the Red-whiskered bulbul (*Pycnonotus jocosus*). A previous study on this species in Reunion Island reported a rapid morphological differentiation between two populations introduced in

different environments (Amiot et al. 2007). Moreover, a second study on these two populations and two additional ones demonstrated that the two populations of Reunion have been founded independently. In addition, a genetic differentiation caused by stochastic demographic processes was identified between the populations of Red-whiskered bulbuls founded independently (Le Gros et al. submitted). These results indicate that the morphological differentiation observed on Reunion was likely caused by stochastic evolution. However, no morphological data were available for the other introduced populations. Here, we thus wanted to describe the morphology of individuals in the other introduced populations, and to assess whether it is linked to the demographic history previously identified, or if other mechanisms could explain it. In addition, we wanted to compare the morphology of individuals in introduced populations and in their source populations to assess the role of the phylogenetic origin of populations on their morphology.

The Red-whiskered bulbul is native of South-eastern Asia and nine subspecies have been described based on coloration patterns and morphology (Peters 1960; del Hoyo et al. 2005). This bird was introduced in many places mainly during the 1960s and 1970s and invasive populations have become established in Australia, the United States (including Hawaii), and islands of the Indian Ocean such as Seychelles, Reunion and Mauritius (Lever 2010). Historical data show that the Red-whiskered bulbul has been introduced in Reunion at the south-eastern point of the island in 1972, probably from Mauritius (Clergeau and Mandon-Dalger 2001; Lever 2010). From there it colonized the eastern and western coasts which are separated by a high mountain range. Because of this mountain range, the climatic conditions are different between the two coasts. The windward coast, exposed to the prevailing wind is more humid than the leeward coast (Amiot et al. 2007). The study by Amiot *et al*. demonstrated a significant difference in beak morphology between the populations of the two coasts. The Red-whiskered bulbul has also been introduced in two other tropical islands about the same size as Reunion and presenting a windward and leeward coast: Mauritius and Oahu (Hawaii). These populations were the one studied in Le Gros et al. (submitted). The population in Oahu was founded in 1965, approximately at the same time as in Reunion whereas Mauritius population was established in 1891. With the study of the Red-whiskered bulbul in these islands, we were able to compare three replicated cases of morphological changes in populations introduced in islands with two contrasted environments (windward and leeward coasts).

Methods

1) Sampling

Morphological measurements and pictures were taken by the same person (ALG) from 612 Red-whiskered bulbuls. Individuals from the native range were measured in the collections of the British Natural History Museum (n=277). Individuals from invasive populations were captured on Reunion (n=241), Mauritius (n=50) and Oahu (n=44). The bulbuls captured on Reunion were caught during a control program organized by the FDGDON (Fédération Départementale des Groupements de Défense contre les Organismes Nuisibles de la Réunion) during 2002 and 2003. They were captured in 12 sites spread along the windward and the leeward coasts (figure 1A). The individuals from Mauritius and Oahu were captured in 2013. They were caught with mist-nets or traps, measured, photographed and released immediately afterward. On Mauritius, bulbuls were caught in three biological stations administrated by an NGO, the Mauritian Wildlife Foundation. One site was on the windward coast and the two others were on the leeward side but in the mountains (figure 1B). On Oahu, the bulbuls were caught in two sites, one on the leeward coast and the other on the windward coast (figure 1C).

For the phylogeographic study, some fresh tissue samples and toe-pads were obtained from museum specimens collected in the native range. Toepads were also sampled on the individuals caught during the control program on Reunion and two feathers were collected on the individuals caught on Mauritius and Oahu. Besides the samples from the introduced populations Reunion, Mauritius and Oahu, we obtained toe-pads from museum specimens collected and in invasive populations of Australia. These samples were used to assess the origin of introduced populations in the world in general. Some sequences from the native range and available in Genbank were also added (table S1). Following the results of Moyle and Marks (2006), *Pycnonotus sinensis*, *Pycnonotus barbatus* and *Pycnonotus cafer* were selected as out-groups.

2) DNA extraction and amplification

DNA was isolated either from toe-pads, the basal part of feathers or blood/muscle samples. Total genomic DNA was extracted with the QIAamp DNA Micro Kit (Qiagen) following the manufacturer instructions for the blood and tissue samples. The digestion volume was doubled, with the final concentration of 2mg/mL for Proteinase K and $2 \cdot 10^{-2}$ mM for dithiothreitol. Two regions of the mitochondrial genes COI and ND2 were amplified (655 bp and 564 bp respectively). For the fresh tissue samples, the two genes were amplified in one fragment whereas in the case of toe-pads samples, short overlapping fragments (200- 300 bp)

were amplified with internal primers (table S2). The amplification protocols used are described in the supplementary material.

3) Phylogeographic analyses

The two genes were concatenated in a partitioned dataset analyzed under the Bayesian inference and the maximum likelihood criteria.

The Bayesian inference was conducted with MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003). In order to account for the potential differences in nucleotide substitution models between the data partitions corresponding to the two genes, a mixed model approach was implemented. MRMODELTEST 2.3 (Nylander 2004) and PAUP* (Swofford 2003) were used to obtain the models best fitting the data, according to the AIC criterion (Akaike 1974). Uniform interval priors were selected for the parameters, except for base frequencies, which were assigned a Dirichlet prior (Huelsenbeck and Ronquist 2001). Two independent runs of four incrementally heated Metropolis-coupled MCMC chains were run for 10 million generations. Sampling was done every 1000 generations, yielding 20000 trees. The online version of AWTY (Nylander et al. 2008) was used to assess the convergence of the MCMC chains and to estimate the "burn-in" length (2000 trees).

Maximum likelihood searches of the partitioned dataset were conducted with RAxML v. 7.0.3 (Stamatakis 2006) using a GTR+Γ+I model and a random starting tree. The α-shape parameters, GTR-rates, and empirical base frequencies were estimated and optimized for each partition. Nodal support was estimated using 100 bootstrap replicates.

4) Morphological data acquisition

Five morphological measurements were recorded on all individuals. Beak length, beak width and beak depth were measured with a digital caliper (to nearest 0.1 mm). Folded wing length and central tail feather length were measured with a metal ruler (to nearest 0.5 mm). To increase precision, all measurements were taken twice and averaged for live individuals. Log-shape ratios were used in order to allow the study of morphological variables independently of size (Mosimann and James 1979). Following this method, the overall size of each individual was defined as the mean of the log-transformed measurements (excluding tail length as it was sometimes missing). Each measurement was then standardized by subtracting the overall size of the individual to the log-transformed measured value.

Pictures in lateral view of the beak of individuals were taken in standardized conditions. TPSDIG 2 (Rohlf 2010a) was used to digitize four landmarks (homologous points)

and 20 semi-landmarks (pseudo-homologous points) from these pictures in order to describe the beak shape. The four landmarks were positioned on the tip of the beak (point 1, figure 2), at the opposite extremity of the beak on the upper and lower mandibles (points 3 and 4, figure 2) and at the anterior extremity of the nostril (point 2, figure 2). Semi-landmarks were spaced equally between the points 1-3 (10 points) and 1-4 (10 points) to describe the beak outline. All pictures were digitized by the same person and the repeatability of the digitization process was tested using a principal components analysis (PCA) on three repetitions taken on five specimens chosen randomly from the same sampling site. Variation was much lower within repetitions than between individuals, indicating the good repeatability of the digitization process (figure S1).

A Generalized Procrustes superimposition (Rohlf and Slice 1990) of the points digitized for each individual was then performed using TPSRELW (Rohlf 2010b). With this method the set of landmarks digitized for each individuals are transformed in order to minimize differences between individuals. This is done by adjusting their position, rotation and scale while conserving the shape they define (Adams et al. 2004). Semi-landmarks are also slid along the curves they describe to match as well as possible the positions of the corresponding points in a reference specimen randomly chosen (Adams et al. 2004). The coordinates obtained after this step are those used for the analysis of shape. The size of the individuals was defined as the log-transformed centroid size.

5) Morphometric analyses

Statistical analyses were done with R 2.15.3 (R Core Team 2013) and using the libraries Ape (Paradis et al. 2004), Hotelling (Curran 2006), and Rmorph (Baylac 2012). PCAs performed separately on the body log-shaped ratios and the Procrustes residuals, were used to summarize the information contained in these two data sets with fewer variables. The principal component axes representing 95% of the total variance were kept as morphological variables for further analyses. Multivariate regressions were performed between size and morphological variables to test for allometric effects. Multivariate analyses of covariance (MANCOVAs) were the performed to assess the differences between individuals in source and introduced populations and between windward and leeward environments. Sex was added as co-factor to control for sexual dimorphism. Size was not added as it was correlated to sex and sampling sites. Hotelling T-squared tests (H tests) were used for pairwise comparison and the threshold of acceptance of the null hypothesis was divided by the number of pairwise comparisons performed following the Bonferonni correction. Finally, neighbor-joining trees based on

Euclidian distances between sampling sites centroids (mean variables values for the individuals in each site) were constructed to visualize the differences between sampling sites.

Results

1) Phylogeography

We obtained sequences for 27 specimens of the native populations, from all currently recognized subspecies, plus 41 individuals sampled from the introduced populations (Reunion 8 individuals, Maurice 8 ind., Oahu 18 ind., Australia 7 ind.). The sequences of three additional specimens were retrieved from Genbank. The two concatenated genes yielded a 1219 bp alignment. The output of MRMODELTEST suggested as the best fit the HKY+Γ and the GTR+I models for the COI and ND2 genes, respectively.

The phylogenetic analysis recovers four main clades (figure 3). The current subspecies subdivision matches rather poorly with the clade subdivision obtained here, but the four clades are coherent with the geographic origin of the specimens (figure 4). The first clade contains the individuals from western and southern India (subspecies *P. j. abuensis* and *fuscicaudatus*). The second clade is composed of individuals from the lowlands of eastern India to western Burma (some but not all *P. j. emeria*, *monticola* and *pyrrhotis*) and Andaman Islands (*P. j. whistleri*). The third clade is composed of individuals from the Himalayas (some but not all *P. j. monticola* and *pyrrhotis*). The individuals from South-eastern Burma, Thailand, China, Laos and Vietnam fall together in a last group (*P. j. jocosus*, *hainanensis*, *pattani* and some but not all *emeria* and *monticola*).

The individuals from Reunion and Mauritius share the same haplotype and they fall in the eastern Indian clade. The majority of Oahu samples also share a single haplotype belonging to the eastern Indian clade, except for two individuals (out of 18) that fall into the Indochinese clade. Also all individuals introduced in Australia belong to the Indochinese clade (figure 3).

2) Morphometry

The first two axes of the PCA performed on the log-shaped ratios explain 74.3% of the total variability (50.3 and 24% respectively). In the morphospace defined with these two axes, centroids the subspecies of the native range and the introduced populations are located in opposite sides (figure 5a). However, the set of points of the two groups are partially superposed (figure S3a). The sites from the three islands are also separated in the morphospace except for the windward site of Mauritius which is located with the sites of

Reunion. The sites within islands are close in the morphospace but not superposed indicating small morphological differences between their individuals. For Reunion and Mauritius, the sites belonging to each coast are grouped together in the morphospace, except for one site of the leeward coast of Reunion which is closer to the sites of the windward coast. This morphological pattern is also visible on the neighbor-joining tree calculated with Euclidian distances between the centroid of each population (figure 5a). There is a significant allometric effect (P<2.2e-16), with a negative correlation between size and the first axis (slope=-5.92.10^{-3}, SE=4.30.10^{-4}) and a positive correlation between size and the fourth axis (slope=4.05.10^{-3}, SE=1.27.10^{-3}). The MANCOVA performed on the first four axes showed that there is a significant morphological difference between the individuals from the native area and introduced population (Wilks test, P< 2.2e-16). Reunion is significantly different from its supposed source, Mauritius (H test, P=1.62e-10). There is also a significant difference between the leeward and windward coasts of Reunion and Mauritius but not of Oahu (table 1). Moreover, there are significant differences among windward sites (except between Mauritius and Reunion) and leeward sites between islands (table 1).

For the PCA performed on the Procrustes residuals, the first two axes explain 82.1% of the total variability (61.4 and 20.7% respectively). Again, the individuals from the source and introduced populations are separated in the morphospace except for the individuals of Oahu which are close to those of the native range (figure 5b). There is also again a partial superposition between the set of points of the native and introduced range (figure S3b). The sites from the three islands are separated in the morphospace. Once again, the sites within islands are close in the morphospace but not superposed indicating small morphological differences between their individuals. However, windward and leeward sites were less well distinguished within islands than in the analysis of log-shaped ratios. The same morphological pattern was obtained with the neighbor-joining tree calculated with Euclidian distances between the centroid of each population (figure 5b).There is a significant allometric effect (P< 2.2e-16), with a positive correlation between size and the first axis (slope=0.39, SE=0.045) and a negative correlation between size and the two other axis (slope=-0.43 and -0.36 respectively, SE=0.078 and 0.10 respectively). The MANCOVA performed on the first three axes showed a significant difference between the native and invasive individuals (Wilks test, P<2.2e-16) even between the individuals from Oahu and the source populations (H test, P=9.22e-06). Reunion is significantly different from its supposed source, Mauritius (H test, P=1.62e-08).There is again a significant morphological difference between the individuals of the windward and leeward coast in Reunion and in Mauritius but not in Oahu (table 1). Again,

there are some significant morphological differences among windward and leeward coasts between islands except for the windward sites of Mauritius and Oahu (table 1).

Discussion

1) Introduced populations come from the same source

Based on mitochondrial data, the Red-whiskered bulbul shows a strong phylogeographic structure, with four mains clade corresponding to four distinct geographic areas: Western and Southern India, lowlands of eastern India and Western Burma, Himalayas and Indochina. However, specimens attributed to a given subspecies are not always included in the same clade, questioning the current subspecific taxonomy of this species. The individuals belonging to introduced populations of Reunion, Mauritius and Oahu all fell in the Indian clade (except for two individuals of Oahu that fell in the Indochinese clade). Interestingly, all individuals introduced in Australia were part of the Indochinese clade. We thus considered that the invasive populations of the Reunion and Oahu we studied were introduced from the same main source but that at least two genetic groups are involved in the invasion worldwide. The fact that all individuals from Reunion and Mauritius share a single haplotype supports the hypothesis that Mauritius is the source of the populations on Reunion. In Oahu, the coexistence of two distinct genetic groups suggests multiple introductions from different sources. However, only two out of 18 sequenced specimens fell into the Indochinese clade. Moreover the study of microsatellite loci did not distinguished them form the other individuals of Oahu (Le Gros et al. submitted). These results indicate a possible introgression between the two genetic groups when they were introduced on Oahu. We supposed that alleles of Indochinese origin were represented but in a minor proportion than Indian alleles in the genotypes of the Red-whiskered bulbuls in Oahu.

2) Morphological differentiation is likely caused by stochastic evolution

The morphological differentiation pattern on log-shaped ratios for the whole body and on Procrustes residuals for the beak shape is quite similar. First the introduced populations and their source are significantly differentiated. This pattern may be explained by a rapid adaptation of introduced populations to new environments but also by phenotypic plasticity or stochastic evolution (*i.e.* founder effect or genetic drift). Secondly, in Reunion and Mauritius, there are significant differences between windward and leeward sites. However, the hypothesis of rapid adaptation to different environments is not supported as there are no significant differences between the windward and leeward site of Oahu. Moreover, there are

also significant differences between a type of coast across islands both in the case of windward and leeward coast. The only exception was the windward sites of Reunion and Mauritius that were not significantly different when log-shaped ratios (but not Procrustes residuals) were compared. This could be explained by the fact that the bulbuls from Reunion are supposed to come from Mauritius (Lever 2010). The source population of Reunion could therefore be on the windward coast of Mauritius.

The study of morphology in these populations showed that (1) the introduced populations although of the same genetic origin are morphologically distinct; (2) the introduced populations are also morphologically distinct from the source population and (3) the morphological differentiation observed within Reunion and Mauritius among contrasted environments is probably not explained by local adaptation as this differentiation was not observed on the third replicate: Oahu. The results of a previous population genetic study (Le Gros et al. submitted) on the same populations showed that the populations of the three islands were genetically different and that there were two genetically distinct populations on Reunion that had been founded independently. These results show that recent demographic history of the populations (i.e. founder effect and possible subsequent bottlenecks) has played a role in their genetic differentiation. As the morphological pattern observed in these three islands corresponds to the genetic structure previously identified, it is likely that the recent demographic history of populations is also the main driver of the morphological differences observed between islands but also within Reunion.

3) A possible cases of rapid adaptation

The only exception is the case of Mauritius where morphological differences exist between the two sides of the island in the absence of genetic structure. In this case, stochastic evolution is therefore not the cause of the morphological differentiation observed. Two possible causes remain to explain it *i.e.* phenotypic plasticity or local adaptation to contrasted environments. If we suppose that the morphological differentiation observed between the two coasts of Mauritius is adaptive, a morphological differentiation should be adaptive too on Oahu. However, there are no significant morphological differences between populations of the two coasts in Oahu where the bulbul has been introduced approximately 50 years ago, and there are some differences in Mauritius where the bulbul has been introduced approximately 120 years ago. In both cases, there is no genetic structure within the island. This supports the hypothesis that adaptive evolution rather than plasticity is causing the morphological differentiations observed, as a morphological differentiation would already be visible on Oahu

otherwise. Under this hypothesis, the selection process has not yet visible consequences on the morphology of individuals in Oahu. With an estimated generation time of Red-whiskered bulbuls between 1.5 and two years, these results mean that the adaptation process takes between 25 and 80 generations to have visible consequences. This time period is longer than the time taken by the bulbul to colonize the whole islands of Reunion and Oahu (Williams and Giddings 1984; Clergeau and Mandon-Dalger 2001). Therefore, even if a rapid local adaptation of the morphology of bulbuls has occurred in the introduced populations we studied, it is unlikely that it has favored their establishment. However, we cannot exclude that rapid adaptation of other traits might have favored their establishment. Moreover, we found evidence indicating that the morphological differentiations observed between islands are likely to result from stochastic evolution. However, it is also possible that selection responsible for a part of these morphological differentiations.

4) Advantages of the comparative approach

Conducting common garden experiments is the renowned way to assess if a change is plastic or heritable (Merilä and Hendry 2014). Similarly reciprocal transplants are widely used to assess if a phenotypic change is adaptive (Hereford 2009). However, conducting these two kinds of experiments is sometimes impossible because of the difficulties of raising some species in captivity, because of legal constraints, or for ethical reasons. Animal models are also a way for evaluating the genetic basis of phenotypic change but they require long-term studies of populations (*e.g.* Charmantier et al. 2008).

In this article, we highlight that comparative studies can be used as a preliminary step in the study of rapid adaptation. Indeed, they allow identifying cases in which rapid adaptation is likely to have occurred when the role of phylogeny and recent demographic history on phenotypic changes can be rejected. This has also been illustrated by Clegg et al. (2002) in a study on a bird species introduced from Australia in several islands. By comparing the morphology of the birds between islands, the authors were able to show that directional natural selection was likely to be the cause of the morphological changes observed in these islands. Complementary studies can then be used to test the hypothesis formulated with the results of this approach. For example, genetic data can also be used to study changes in genes known to influence the studied phenotypic trait (Corre and Kremer 2003). If there is no information available on the genetic basis of the trait considered, an alternative method to assess if some parts of the genome are under selection, can be to use genome scans based on

anonymous loci and to look for loci that exhibit unusually large genetic distances between populations (*e.g*. Kayser et al. 2003).

Conclusion

The comparative approach used in this paper allowed us to see that there was a morphological differentiation between introduced populations and their source. However there was not always a differentiation between contrasted environments within islands. We thus attributed this differentiation to stochastic evolution. This was confirmed by the results of a previous study on population genetics except in the case of Mauritius. Indeed, we found that rapid adaptation might have occurred on this island. However if this hypothesis is true, we estimated that is unlikely to have favored the establishment of the bulbuls on Mauritius. Finally, we argue that using a comparative approach is a first step in the study of rapid adaptation as it allows the rejection of alternative hypotheses which are easier to test.

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Table 1: Results of the pairwise comparisons between populations conducted with Hotelling's T-squared tests.

Populations compared	Hotelling's T^2 statistic	scaling factor	df	nx	ny	P-value
Log-shaped ratios						
Reunion W/L	29.80	0.25	4; 222	157	70	1.41e-05*
Mauritius W/L	29.02	0.23	4; 45	12	38	2.27e-04*
Oahu W/L	3.77	0.23	4; 39	10	34	0.49
Windward R/M	3.37	0.25	4; 164	157	12	0.51
Windward R/O	41.70	0.25	4; 162	157	10	2.06e-07*
Windward M/O	35.63	0.21	4; 17	12	10	1.08e-03*
Leeward R/M	121.11	0.24	4; 103	70	38	2.22e-16*
Leeward R/O	138.33	0.24	4; 99	70	34	0.00*
Leeward M/O	34.31	0.24	4; 67	38	34	1.89e-05*
Procrustes residuals						
Reunion W/L	31.75	0.25	4; 242	172	75	5.88e-06*
Mauritius W/L	12.15	0.23	4; 43	12	36	3.56e-02~
Oahu W/L	7.93	0.23	4; 37	9	33	0,14
Windward R/M	15.96	0.25	4; 179	172	12	4.44e-03*
Windward R/O	36.37	0.25	4; 176	172	9	1.35e-06*
Windward M/O	18.40	0.21	4; 16	12	9	2.19e-02~
Leeward R/M	41.32	0.24	4; 106	75	36	6.22-07*
Leeward R/O	100.98	0.24	4; 103	75	33	2.82e-14*
Leeward M/O	25.27	0.24	4; 64	36	33	3.49e-03*

*Indicates a significant difference between populations compared. The significance threshold of 0.05 was divided by three to account for multiple testing following the Bonferroni correction. ~Indicates a difference close to significance.

Figures

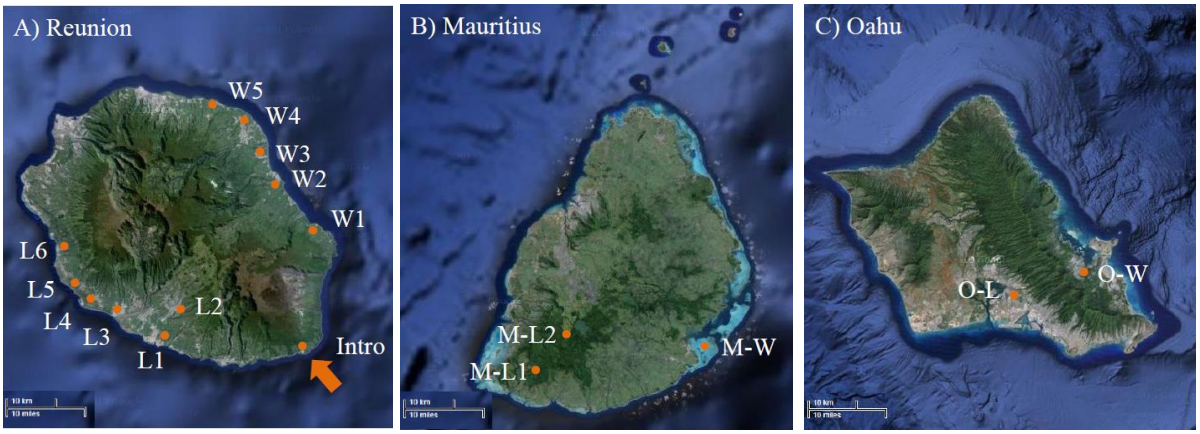

Fig. 1: Sampling sites on Reunion, Mauritius and Oahu (Hawaii). The arrow shows the location on Reunion where the Red-whiskered bulbul was introduction in 1972.

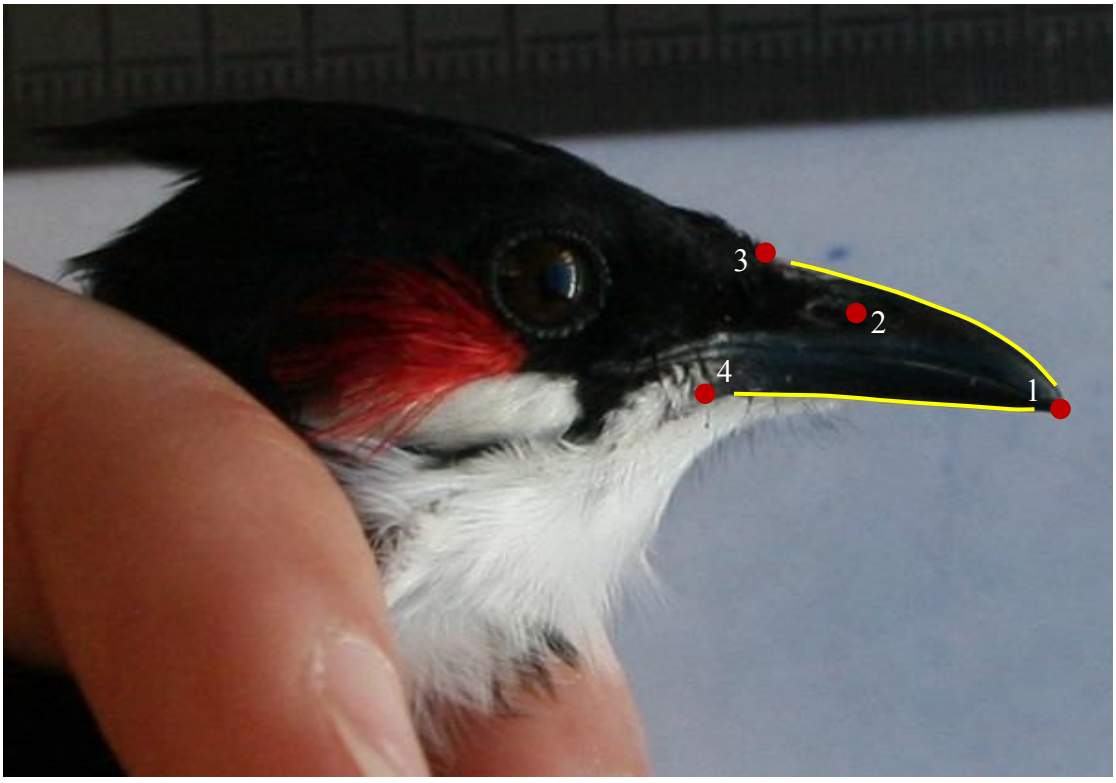

Fig. 2: Position of the four landmarks and the two outline curves (10 semi-landmarks equally spaced for each) digitized on beak pictures.

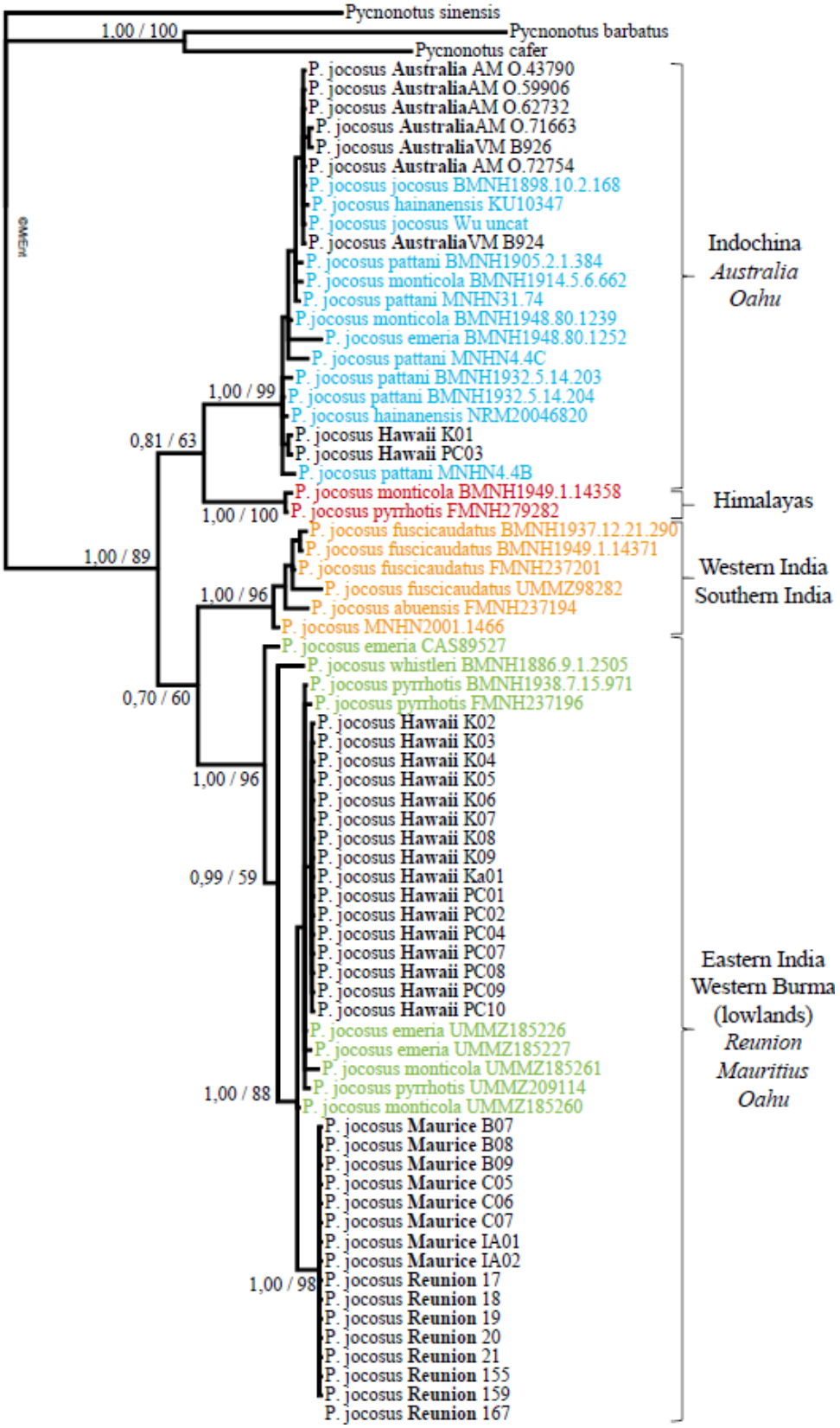

Fig. 3: The majority rule consensus tree obtained from the mixed-model Bayesian analysis of the concatenated dataset. The support values indicated at the node are the posterior probability (threshold 0.50) and the bootstrap support (threshold 50%) obtained from the maximum likelihood analysis, respectively. Colors indicate the geographic origin of the individuals (see figure 4).

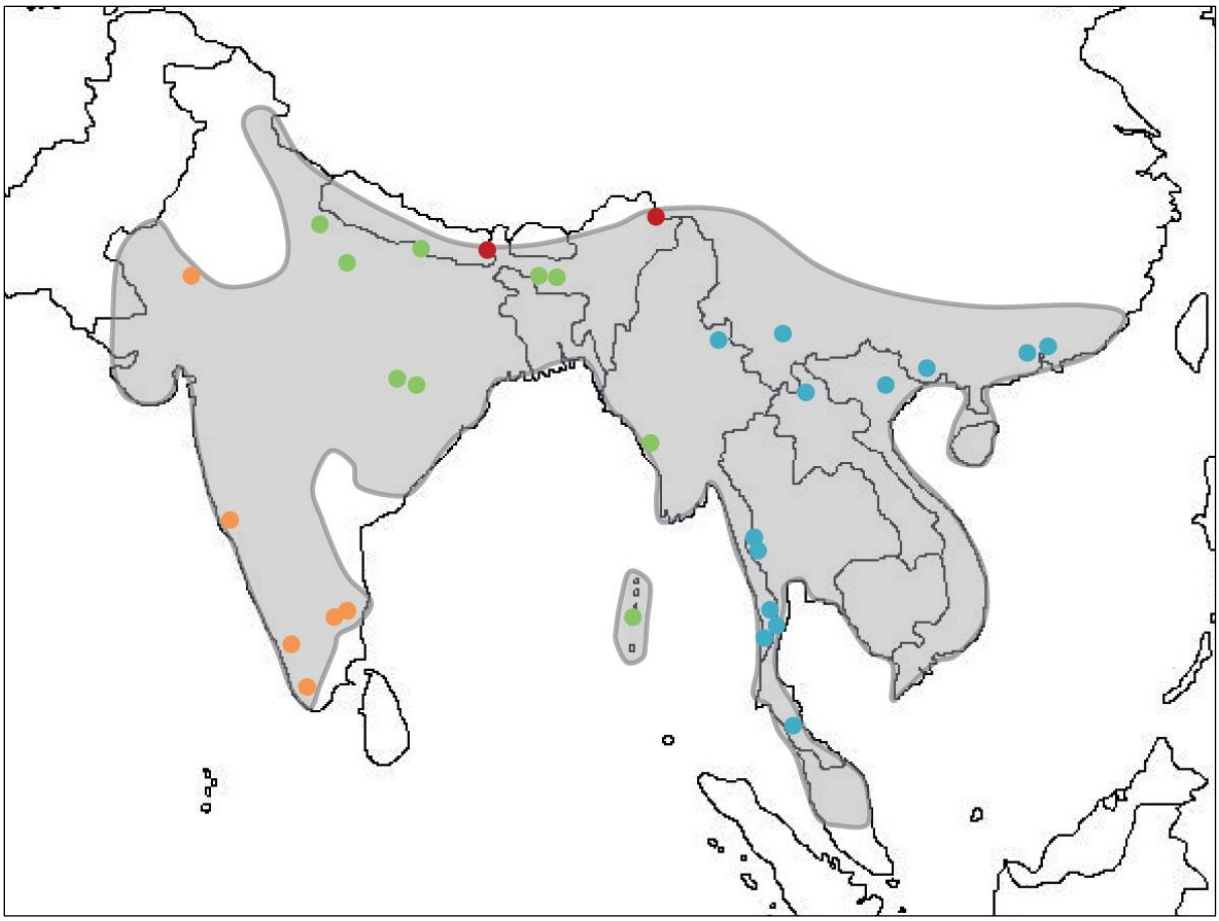

Fig. 4: Native range of the Red-whiskered bulbul (in grey), and location of the specimens used in the phylogeographic analysis. Colors indicate the clade to which each sample belongs; orange: Western and Southern India, green: lowlands of Eastern India and Western Burma; red: Himalayas, and blue: Indochina.

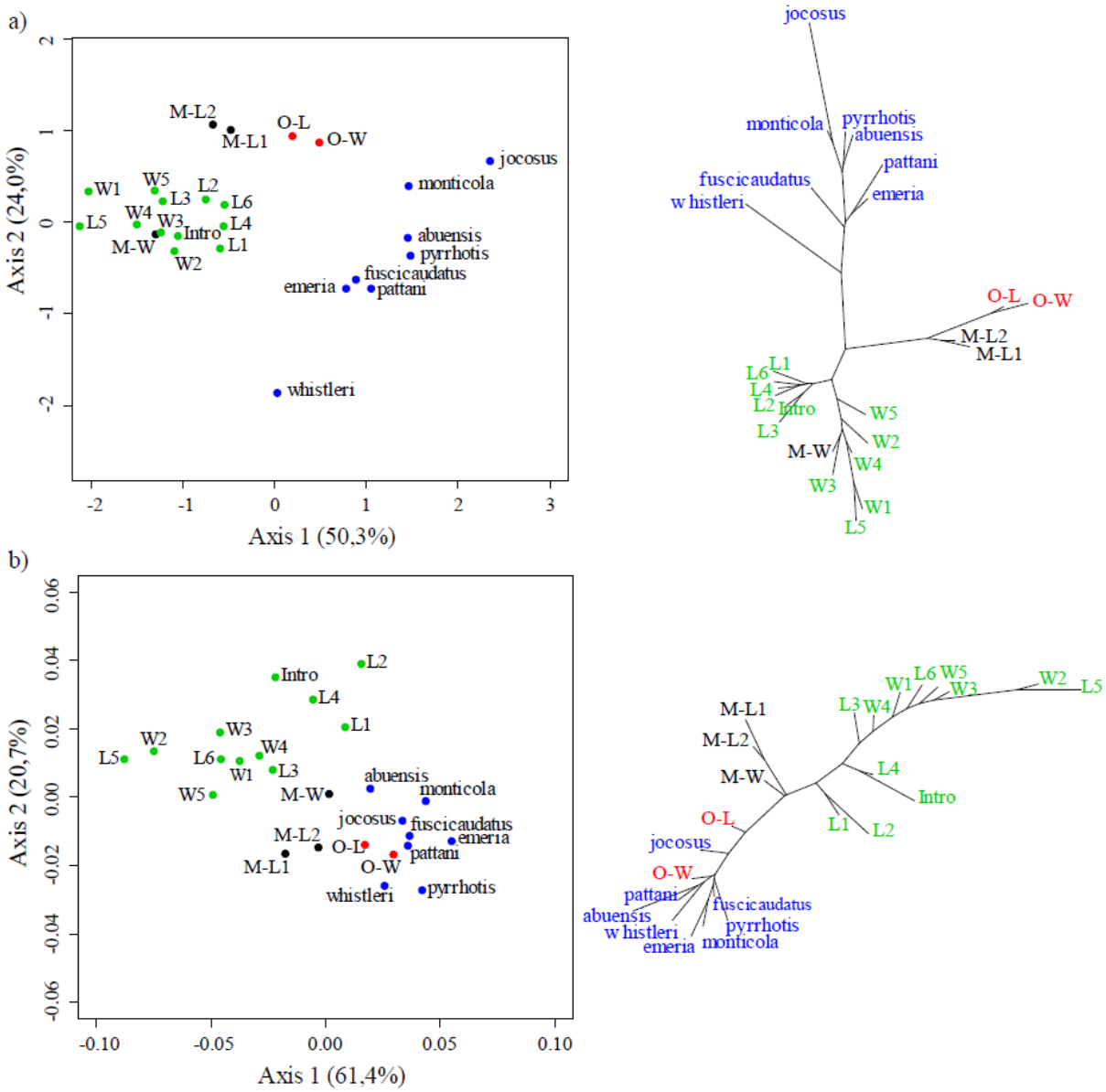

Fig. 5: Left: centroid of each subspecies or introduced population plotted on the first two axes of the PCA performed on a) log-shaped ratios, and b) Procrustes residuals. Right: neighbor-joining trees calculated with the Euclidian distance between the centroid of each subspecies or introduced population for a) log-shaped ratios, and b) Procrustes residuals. Blue: native range, green: Reunion, black: Mauritius and red: Oahu. Correlation circles and conformations associated to the axes of the PCAs are in the supplementary material (figures S3 and S4).

Supplementary Material

1) Amplification protocols

The following reagent quantities were used to amplify the COI and ND2 genes in a single fragment: 13.94µL of Milli-Q water, 2µL of polymerase buffer 10x (Qiagen), 1.5µL of $MgCl_2$ (Qiagen, 25mM), 1µL of Dimethyl sulfoxide (DMSO), 0.8µL of dNTPs mix (1.7mM each), 0.32µL of each primer (10µM), 0.12µL of DNA polymerase (Qiagen, Taq 5 units/µL). Cycling conditions: 94°C, 5 min.; (94°C, 40 sec.; 55°C, 40 sec.; 72°C, 60sec.) x 40 cycles; 72°C, 5 min.

For the amplification of short fragments, illustra™ Hot Start Mixes (GE Healthcare) were used with the following reagent quantities: 19µl of Milli-Q water, 1µL of $MgCl_2$ (Qiagen, 25mM), 1µL of each primer (10µM). Cycling conditions: 94°C, 5 min.; (94°C, 40 sec.; 61°C, 40 sec.; 72°C, 60sec.) x 4 cycles; (94°C, 40 sec.; 59°C, 40 sec.; 72°C, 60sec.) x 4 cycles; (94°C, 40 sec.; 57°C, 40 sec.; 72°C, 60sec.) x 32 cycles; 72°C, 5 min.

2) Supplementary tables

Table S1: details of the specimens used in the phylogeographic analysis.

Species	Subspecies	Country	Locality	ID Number	COI	ND2
P. jocosus	jocosus	China	South China	BMNH 98.10.2.168	unpub ALG	unpub ALG
P. jocosus	whistleri	India	Andaman islands	BMNH 86.9.1.2505	unpub ALG	unpub ALG
P. jocosus	pyrrhotis	Nepal	NA	BMNH 1938.7.15.971	unpub ALG	unpub ALG
P. jocosus	fuscicaudatus	India	South Madras	BMNH 1949.1.14371	unpub ALG	unpub ALG
P. jocosus	fuscicaudatus	India	South Madras	BMNH 1937.12.21.290	unpub ALG	unpub ALG
P. jocosus	emeria	Myanmar	Lower Myanmar	BMNH 1948.80.1252	unpub ALG	unpub ALG
P. jocosus	monticola	India	Upper Assam	BMNH 1949.1.14358	unpub ALG	unpub ALG
P. jocosus	monticola	China	Yunnan	BMNH 1914.5.6.662	unpub ALG	unpub ALG
P. jocosus	monticola	Myanmar	Upper Myanmar	BMNH 1948.80.1239	unpub ALG	unpub ALG
P. jocosus	pattani	Myanmar	South Tennasserim	BMNH 1932.5.14.203	unpub ALG	unpub ALG
P. jocosus	pattani	Myanmar	South Tennasserim	BMNH 1932.5.14.204	unpub ALG	unpub ALG
P. jocosus	pattani	Malaysia	North penninsular Malaysia	BMNH 1905.2.1.384	unpub ALG	unpub ALG
P. jocosus	pattani	Laos	Boun Tai	MNHN 31-74	unpub ALG	unpub ALG
P. jocosus	pattani	Thaïlande	Umphang	MNHN 04-4C	unpub ALG	unpub ALG
P. jocosus	pattani	Thaïlande	Umphang	MNHN 04-4B	unpub ALG	unpub ALG
P. jocosus	NA	Inde	NA	MNHN 2001-1466	unpub ALG	unpub ALG
P. jocosus	emeria	Myanmar	Rakhaine State, Gwa Township	CAS 89527	unpub ALG	unpub ALG
P. jocosus	abuensis	India	Rajasthan	FMNH 237194	unpub ALG	unpub ALG
P. jocosus	fuscicaudatus	India	Kerala	FMNH 237201	unpub ALG	unpub ALG
P. jocosus	pyrrhotis	India	Uttar Pradesh	FMNH 237196	unpub ALG	unpub ALG

P. jocosus	pyrrhotis	Nepal	NA	FMNH 279282	unpub ALG	unpub ALG
P. jocosus	emeria	India	Madhya Pradesh, Mandla District	UMMZ 185226	unpub ALG	unpub ALG
P. jocosus	emeria	India	Madhya Pradesh, Mandla District	UMMZ 185227	unpub ALG	unpub ALG
P. jocosus	fuscicaudatus	India	Karnataka, Belgaum District	UMMZ 98282	unpub ALG	unpub ALG
P. jocosus	pyrrhotis	India	Uttar Pradesh	UMMZ 209114	unpub ALG	unpub ALG
P. jocosus	monticola	India	Assam, Goalpara District	UMMZ 185260	unpub ALG	unpub ALG
P. jocosus	monticola	India	Assam, Goalpara District	UMMZ 185261	unpub ALG	unpub ALG
P. jocosus	NA	France (Reunion)	Saint-Benoît	Clergeau 17	unpub ALG	unpub ALG
P. jocosus	NA	France (Reunion)	Saint-Benoît	Clergeau 18	unpub ALG	unpub ALG
P. jocosus	NA	France (Reunion)	Saint-Benoît	Clergeau 19	unpub ALG	unpub ALG
P. jocosus	NA	France (Reunion)	Saint-Benoît	Clergeau 20	unpub ALG	unpub ALG
P. jocosus	NA	France (Reunion)	Saint-Benoît	Clergeau 21	unpub ALG	unpub ALG
P. jocosus	NA	France (Reunion)	Les Avirons	Clergeau 155	unpub ALG	unpub ALG
P. jocosus	NA	France (Reunion)	Les Avirons	Clergeau 159	unpub ALG	unpub ALG
P. jocosus	NA	France (Reunion)	Les Avirons	Clergeau 167	unpub ALG	unpub ALG
P. jocosus	NA	Mauritius	Ile aux aigrettes	Le Gros IA01	unpub ALG	unpub ALG
P. jocosus	NA	Mauritius	Ile aux aigrettes	Le Gros IA02	unpub ALG	unpub ALG
P. jocosus	NA	Mauritius	Camp	Le Gros C05	unpub ALG	unpub ALG
P. jocosus	NA	Mauritius	Camp	Le Gros C06	unpub ALG	unpub ALG
P. jocosus	NA	Mauritius	Camp	Le Gros C07	unpub ALG	unpub ALG
P. jocosus	NA	Mauritius	Bel Ombre	Le Gros B07	unpub ALG	unpub ALG
P. jocosus	NA	Mauritius	Bel Ombre	Le Gros B08	unpub ALG	unpub ALG
P. jocosus	NA	Mauritius	Bel Ombre	Le Gros B09	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Pearl City	Le Gros PC01	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Pearl City	Le Gros PC02	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Pearl City	Le Gros PC03	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Pearl City	Le Gros PC04	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Pearl City	Le Gros PC07	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Pearl City	Le Gros PC08	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Pearl City	Le Gros PC09	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Pearl City	Le Gros PC10	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Kailula	Le Gros Ka01	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Kaneohe	Le Gros K01	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Kaneohe	Le Gros K02	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Kaneohe	Le Gros K03	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Kaneohe	Le Gros K04	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Kaneohe	Le Gros K05	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Kaneohe	Le Gros K06	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Kaneohe	Le Gros K07	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Kaneohe	Le Gros K08	unpub ALG	unpub ALG
P. jocosus	NA	USA (Hawaii)	Kaneohe	Le Gros K09	unpub ALG	unpub ALG
P. jocosus	NA	Australia	NA	AM O.72754	unpub ALG	unpub ALG
P. jocosus	NA	Australia	New South Wales, Richmond	AM O.71663	unpub ALG	unpub ALG
P. jocosus	NA	Australia	New South Wales, Figtree	AM O.59906	unpub ALG	unpub ALG
P. jocosus	NA	Australia	New South Wales, Sydney	AM O.43790	unpub ALG	unpub ALG
P. jocosus	NA	Australia	New South Wales, Sydney	AM O.62732	unpub ALG	unpub ALG

P. jocosus	NA	Australia	New South Wales	MV B926	unpub ALG	unpub ALG
P. jocosus	NA	Australia	New South Wales	MV B924	unpub ALG	unpub ALG
P. jocosus	jocosus	China	Guangzhou market	Wu	GU170351	GU170352
P. jocosus	hainanensis	China	Shiwandashan National NP	KU 10347	missing	GU112670
P. jocosus	hainanensis	Vietnam	Hanoi market	NRM 20046820	unpub ALG	GQ242077
P. sinensis	NA	NA	NA	T5464	HQ700433	HQ700401
P. barbatus	NA	NA	NA	USNM 630912	JQ176056	missing
P. barbatus	NA	NA	NA	MNHN 02-29	missing	GQ369695
P. cafer	NA	NA	NA	NA	missing	KJ455616
P. cafer	NA	NA	NA	USNM 620456	JQ176062	missing

Table S2: primers used for amplification of COI and ND2 genes.

Gene	Primer F		Primer R	
COI (single fragment)	COI-ExtF	ACGCTTTAACACTCAGCCATCTTACC	COI-BirdR1	ACGTGGGAGATAATTCCAAATCCTG
COI (1st fragment)	COI-ExtF1b	GATGAYTATTTTCAACCAACCACAAAGA	COI-R220b	CTYATGTTGTTTATTCGRGGGAAAGC
COI (2nd fragment)	COI-F167b	TTGGCGGATTYGGAAACTGACTAGT	COI-R451b	TGTGATAGGGCKGGGGGTTTTATGTT
COI (3rd fragment)	COI-F403b	GGTRTCTCCTCAATCTTAGGAGCAAT	COI-R661b	GGTAGGATTAGGATATAGACTTCTGGATG
ND2 (single fragment)	ND2-ExtF	AGCTATCGGGCCCATACCCCGAA	ND2-ExtR	TTGAAGGCCTTCGGTTTAGGTGA
ND2 (1st fragment)	ND2-ExtF	AGCTATCGGGCCCATACCCCGAA	ND2-R223	TGTCCAGTGTACCATGCGTTGGTCA
ND2 (2nd fragment)	ND2-F175	ACTTCTTGACCCAAGCAACAGCCTCA	ND2-R405	GAATAGTAGCGTGATTGGGGGGAATT
ND2 (3rd fragment)	ND2-F355	TGCAAGGATCCCCCCTTATyACTGGA	ND2-R568	GGGGTTGTAGGTGATGATGATGGCTA

3) Supplementary figures

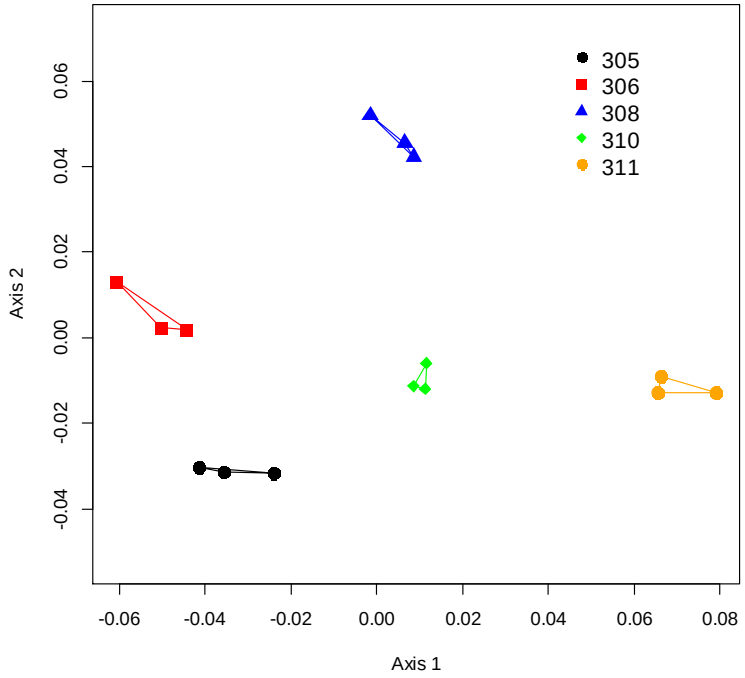

Fig. S1: PCA performed on beak conformation to test the repeatability of the digitization process. Each color represents an individual. Three repetitions where done for each of them.

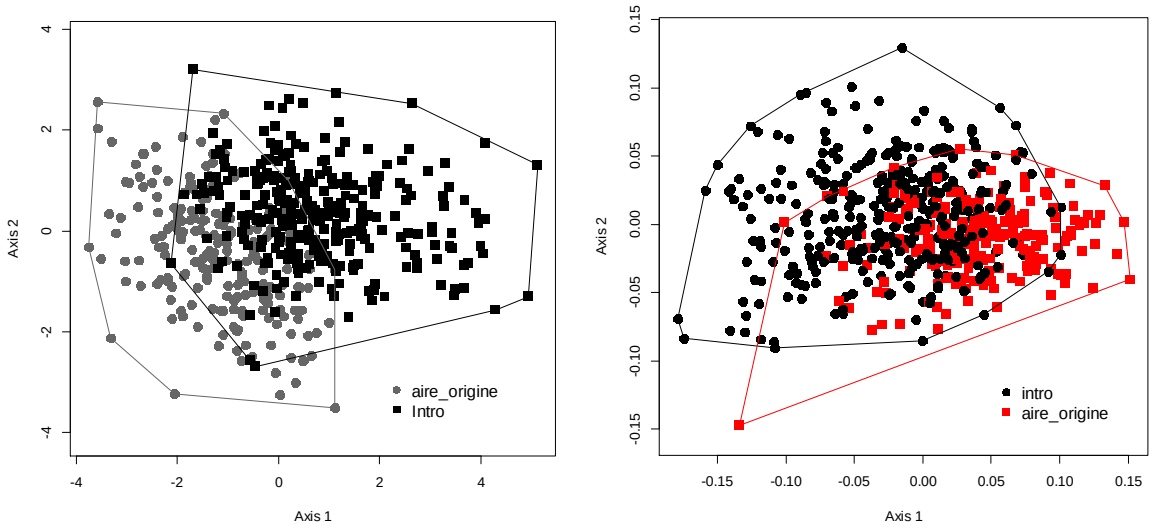

Fig. S2: set of points for the native range (left: grey, right: black) and the introduced range (left: black, right: red) plotted on the first two axes of the PCAs on a) log-shapes ratios, and b) Procrustes residuals

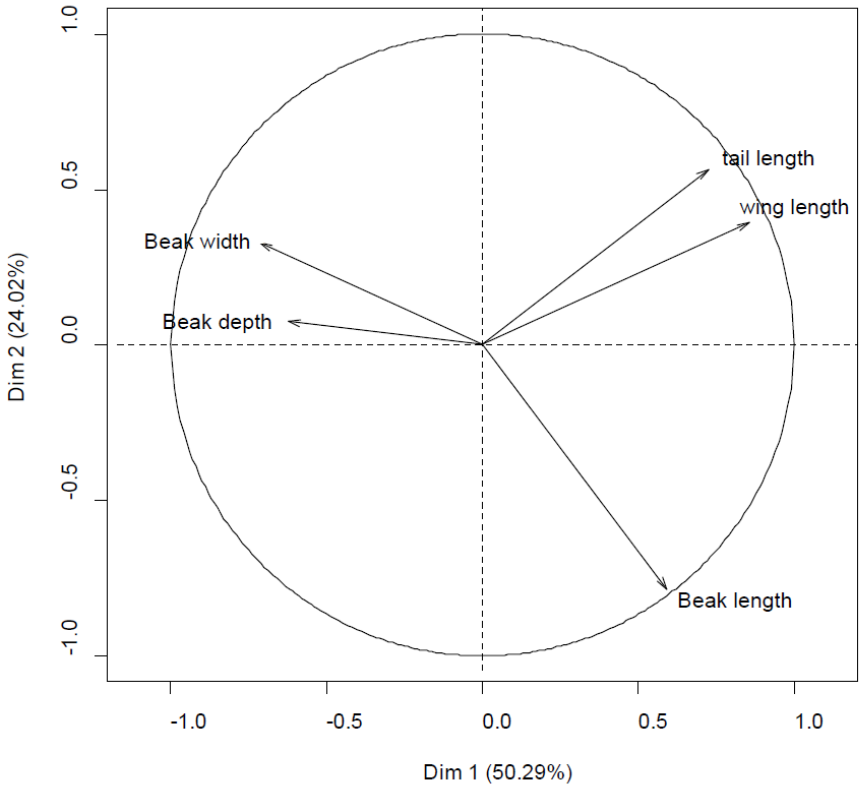

Fig. S3: correlation circle for the PCA on log-shaped ratios.

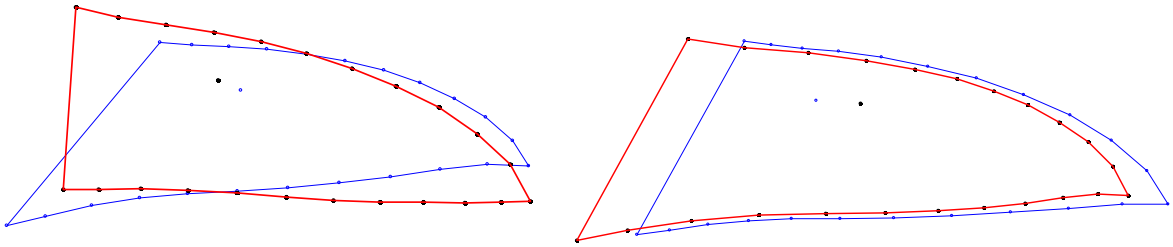

Fig. S4: Beak conformations associated to the first two axes of the PCA on Procrustes residuals. The red conformation corresponds to the positive extremity of the axis and the bule conformation corresponds to the negative extremity.

Manuscript 3

In preparation

Stochastic evolution, rather than rapid adaptation, causes phenotypic differentiation among populations of Ring-necked parakeets, an exotic invasive species introduced to Europe

Ariane Le Gros[1, 2, 3*], Dario Zuccon[2], Raphaël Cornette[2], Michael P. Braun[4], Juan Carlos Senar[5], Sarah Samadi[2] and Philippe Clergeau[3]

[1]Université Paris Diderot, Sorbonne Paris Cité, 75013 Paris, France
[2]Muséum National d'Histoire Naturelle, Département Systématique et Evolution, UMR 7205 ISYEB, 75005 Paris, France
[3]Muséum National d'Histoire Naturelle, Département Ecologie et Gestion de la Biodiversité, UMR 7204 CESCO, 75005 Paris, France
[4]Heidelberg University, Institute of Pharmacy and Molecular Biotechnology, Department of Biology, D-69120 Heidelberg, Germany
[5]Museu de Ciencies Naturals de Barcelona, Evolutionary and Behavioural Ecology Associate Research Unit, CSIC, 0800 Barcelona, Spain
*Corresponding author: Ariane Le Gros, alegros@mnhn.fr

Abstract

Although it is native of tropical regions, the Ring-necked parakeet (*Psittacula krameri*) is a very successful invasive species in Europe. Identifying the factors that favored the establishment of the Ring-necked parakeet would allow a better understanding a management of this species. We were thus interested in knowing if rapid adaptation could explain the establishment of the Ring-necked parakeet in Europe. Experimentally testing this hypothesis was not possible for several reasons. We thus compared the morphology of individuals of native and introduced populations of Ring-necked parakeets to identify cases of rapid phenotypic differentiation. We then assessed if these phenotypic changes could be explain by historical factors (*i.e.* phylogeny and recent demography). In the cases for which these two factors can be rejected, rapid adaptation is then a possible explanation for the phenotypic changes observed. Our phylogeographic analysis shows that the invasive populations we studied come from Asia. Despite their common origin, we found differences between introduced populations even those established in similar environments. Finally, our population genetics analysis shows a genetic differentiation between the introduced populations suggesting that stochastic evolution caused by recent demographic processes has

occurred in these populations. The correspondence between the morphological and genetic differentiations and the morphological differences found between populations introduced in similar environment led us to conclude that stochastic evolution is more likely to be the cause of the morphological differentiation observed than rapid adaptation. This article thus brings new elements in the understanding of the establishment of Ring-necked parakeets in temperate regions.

Key-words: rapid adaptation, invasive species, phylogeography, morphometry, population genetics, *Psittacula krameri*.

Introduction

The Ring-necked parakeet (*Psittacula krameri*) is native from the Indian subcontinent and sub-Saharan Africa where it mainly lives in warm climates. In Asia, its range however reaches the base of the Himalayas (up to 1600 m, Parr and Juniper 2010) indicating a tolerance for colder climates (Thabethe et al. 2013). It is found in a variety of woodlands but also in savanna grassland, farmland and parks and gardens in urban areas (Parr and Juniper 2010). Four sub-species have been described, two in Asia and two in Africa, based on color and size differences (del Hoyo et al. 1997). In Asia, the subspecies *P. k. manillensis* is found below the 20th parallel north in India and in Sri Lanka whereas the subspecies *P. k. borealis* occupies the northern part of the Indian subcontinent. In Africa, the Ring-necked parakeet is found roughly between the equator and the 20th parallel north from the eastern to the western coast. The subspecies *P. k. krameri* occupies the eastern part of this range and the subspecies *P. k. parvirostris* the western part.

The Ring-necked parakeet is a popular cage bird and has been introduced accidentally in many countries since the 1960s. In Europe, populations of Ring-necked parakeets became established in Belgium (1966), the Netherlands (1968), Great Britain (1969), Germany (1969), France (1970s), Italy (1970s), Spain (1982), Portugal (1986), and Greece (1992, Braun 2009). These introduced populations have expanded rapidly and the total European population was estimated at 29,000 individuals in 2008 (Braun 2009). The origin of these introduced populations is not clearly established. Morphological measurements of individuals caught in the United Kingdom suggest that introduced individuals are more similar to the Asian subspecies *P. k. borealis* whereas their beak coloration suggests a closer relationship to the Asian subspecies *P. k. manillensis* (Butler 2003). However, CITES data show that an

equal proportion of Ring-necked parakeets has been imported from Asia and Africa (CITES 2013, table S1).

The impacts of the Ring-necked parakeet in Europe are still under investigation. The species is considered as a major agricultural pest in its native range (Ahmad et al. 2011, 2012) and there are already concerns about its potential economic impacts in Europe, where it is known to feed on buds, flowers, fruits and seeds of trees (Clergeau and Vergnes 2011; Braun and Wink 2013). However, only a few cases of damages on vineyards and orchards have been reported in Europe (Lever 2010). Ring-necked parakeets have also been reported to dig nesting cavities in thermal insulation on buildings (Braun and Wink 2013). Besides this, its presence in suburban areas could have impacts on human health as this species carries the pathogen responsible for the psittacosis disease (Madani and Peighambari 2013). There are also serious concerns about its potential impacts on native species. The parakeet is a cavity nester and starts breeding early in spring, factors that might promote the competition with other species for tree cavities. Two studies have showed a negative correlation between the presence of parakeets and the density of the Eurasian Nuthatches (*Sitta europaea*) in Belgium (Strubbe and Matthysen 2007, 2009b). However, there was no effect on other cavity-nesters (Strubbe and Matthysen 2007). Moreover, three other studies on the nuthatches and the European Starlings (*Sturnus vulgaris*) in Belgium, United Kingdom and Germany did not detect any impact of parakeets on these species (Strubbe et al. 2010; Czajka et al. 2011; Newson et al. 2011). More recently, Hernández-Brito et al. (2014) showed a negative correlation between the presence of parakeets and the Greater noctule bat (*Nyctalus lasiopterus*), an endangered species. Direct aggressive behaviors towards several native species have also been reported (reviewed in Menchetti et al. 2014).

Because of these potential impacts, there might be a need to manage this species in the future. Understanding if introduced Ring-necked parakeets have adapted to local conditions in Europe can be useful in their management (Hendry et al. 2011; Lankau et al. 2011). Indeed, the hypothesis that rapid adaptation can favor the establishment of invasive species in new environment has emerged recently (Sakai et al. 2001; Lee 2002; Lambrinos 2004; Prentis et al. 2008) and might explain why tropical birds have established in a temperate region. We were thus interested in knowing if rapid adaptation has occurred in populations introduced in Europe. In order to identify cases in which rapid adaptation has possibly occurred, we studied four populations introduced in three different climatic regions: the population of Barcelona (Spain, Mediterranean climate), two populations around Paris (France, oceanic climate), the population of Heidelberg (Germany, semi-continental climate). Historical data suggest that all

populations have been founded independently and approximately at the same time. The populations of Paris were founded in the mid-1970s following accidental releases at the two airports (Clergeau et al. 2009). The population of Barcelona was founded in 1982 (Braun 2009). Finally the population of Heidelberg was founded in 1990 but from an older close-by population founded in 1974 (Braun 2009).

Classical ways of testing adaptation are common garden experiments, reciprocal transplants and animal models (Merilä and Hendry 2014). However, common gardens experiments require the raising of wild individuals in captivity and this is not always possible for practical or ethical reasons. Data acquisition following reciprocal transplants requires the follow up of individuals transplanted and their offspring, and there are also legal and ethical problems associated with the transplantation and release of some species into the wild. Finally, animal models allow assessing the heritability of a trait but require data on many individuals with known pedigrees. Long term follow-ups of populations with mark and recapture methods are thus needed to use these models (*e.g.* Charmantier et al. 2008). Here, we used a different approach in which we identified possible cases of rapid adaptation by describing phenotypic differences between populations introduced recently in different environments and by testing alternative hypotheses to rapid adaptation that could explain these differences. Indeed, the phenotypic differences observed can be caused by rapid adaptation to local conditions but also by phenotypic plasticity or by historical factors (*i.e.* phylogeny and recent demographic history). If we can reject some of these alternative hypotheses, then the phenotypic changes observed are more likely to be the result of rapid adaptation.

The phenotypic character we chose to study is the beak and wing morphology of individuals. Indeed, morphology is known to evolve with changes in environmental conditions. For example, beak shape has been showed to evolve with changes in diet (*e.g.* Boag and Grant 1981; Herrel et al. 2005), and wing shape with changes in vegetation (Desrochers 2010). Moreover, contrary to physiological or behavioral data, morphological data can be acquired on museum specimens enabling us to study individuals from the native range of Ring-necked parakeets.

Our approach consisted in comparing the morphology of individuals between different introduced populations. In order to assess the possible cause of the differences observed, we then used (1) a phylogeographic approach to assess whether the phylogenetic origin of populations could explain their phenotype, (2) a population genetics approach to assess the role of recent demographic history of populations on the phenotypic differences observed.

Methods

1) Sampling

For the phylogeographic study, toe-pads were obtained from museum specimens collected in the native range. Some sequences available on Genbank were also added to cover the whole species distribution (table S2). Feathers were obtained from individuals of four introduced populations: two populations near Paris (15 Km to the North-east and 10 Km to the South of Paris, France), one population in Barcelona (Spain), and one population in Heidelberg (Germany), and a captive stock near Bordeaux (France). *Psittacula echo*, *P. columboides*, *P. eupatria*, *P. longicauda*, *P. Alexandri*, *P. cyanocephala*, *P. himalayana*, *P. roseata*, *Tanygnathus sumatranus* and *Eclectus roratus* were used as out-groups.

For the morphological study, measurements were taken on about 400 Ring-necked parakeets by the same person (ALG). Individuals from the native range were measured in the collections of the Muséum National d'Histoire Naturelle and the British Museum of Natural History (*P. k. krameri*=105, *P. k. parvirostris*=14, *P. k. manillensis*=63, *P. k. borealis*=130). Individuals from invasive populations were captured in the North of Paris (Villepinte, n=44), and in the South of Paris (Antony, Chatenay-Malabry, Sceaux, n=56). Standardized pictures of the beak of all these individuals were also taken by the same person (ALG). In addition, we obtained pictures from individuals of the introduced populations in Heidelberg (n=33) and Barcelona (n=10), and from a captive stock near Bordeaux (n=14). Live individuals were caught with traps or at the nest. They were measured, photographed and released immediately afterward.

For the population genetics study, DNA samples were obtained from 10 sample sites: North of Paris (Villepinte n=44; Roissy n=1), South of Paris (Antony n=25; Chatenay-Malabry n=22; Sceaux n=2), Heidelberg (n=30), Barcelona (n=40), Marseille (n=3), Alger (n=3), and the captive stock previously mentioned (n=19).

2) DNA extraction and amplification

Total genomic DNA was isolated from toe-pads and the basal part of feathers with the QIAamp DNA Micro Kit (Qiagen) following the manufacturer instructions for the blood and tissue samples. The digestion volume was doubled, with the final concentration of 2-3 mg/mL for Proteinase K and $2 \cdot 10^{-2}$ mM for dithiothreitol. For the phylogeographic study, a region of the mitochondrial gene cytochrome *b* was amplified (797 bp). For fresh tissues, the gene was amplified in one fragment whereas in the case of toe-pad samples, short overlapping fragments (200- 300 bp) were amplified with internal primers (table S3). The amplification

protocols used are described in the supplementary material. For the population genetics study, the microsatellite loci described in Raisin et al. (2009) were used except for Peq07, Peq16 and Peq21 for which we had problems of amplification. The amplification protocols used are described in the supplementary material. Samples were genotyped on an Applied Biosystems 3130XL DNA sequencer. Genotypes were scored with GeneMapper 4.0 (Applied Biosystems) and checked manually. Living individuals were sexed using the PCR-based protocol of Griffiths et al. (1998), whereas for the museum specimens we relied on the information available on the specimen labels.

3) Morphological data acquisition

Six morphological measurements were recorded on all individuals. Upper mandible length (mm, two measurements), upper mandible width (mm) and upper mandible depth (mm) and cranium length (mm), were measured with a digital caliper (to nearest 0.1 mm, figure S1). Folded wing length (mm) was measured with a metal ruler (to nearest 0.5 mm). To increase precision, all measurements were taken twice and averaged for live individuals. Log-shape ratios were used in order to allow the study of morphological variables independently of size (Mosimann and James 1979). Following this method, the overall size of each individual was defined as the mean of the log-transformed measurements. Each measurement was then standardized by subtracting the overall size of the individual to the log-transformed measured value.

Pictures in lateral view of the beak of individuals were taken in standardized conditions. TPSDIG 2 (Rohlf 2010a) was used to digitize four landmarks (homologous points) and 21 semi-landmarks (pseudo-homologous points) from these pictures in order to describe the beak shape (figure S1). All pictures were digitized by the same person and the repeatability of the digitization process was tested using a principal components analysis (PCA) on three repetitions taken on five specimens chosen randomly from the same sampling site. Variation was much lower within repetitions than between individuals, indicating the good repeatability of the digitization process (figure S2).

A Generalized Procrustes superimposition (Rohlf and Slice 1990) of the points digitized for each individual was then performed using TPSRELW (Rohlf 2010b). With this method the set of landmarks digitized for each individuals are transformed in order to minimize differences between individuals. This is done by adjusting their position, rotation and scale while conserving the shape they define (Adams et al. 2004). Semi-landmarks are also slid along the curves they describe to match as well as possible the positions of the

corresponding points in a reference specimen randomly chosen (Adams et al. 2004). The coordinates obtained after this step are those used for the analysis of shape. The size of the individuals was defined as the log-transformed centroid size.

4) Morphometrics analyses

Statistical analyses were done with R 2.15.3 (R Core Team 2013) and using the libraries Ape (Paradis et al. 2004), Hotelling (Curran 2006), and Rmorph (Baylac 2012). PCAs performed separately on the body log-shaped ratios and the Procrustes residuals, were used to summarize the information contained in these two data sets with fewer variables. The principal component scores representing 95% of the total variance were kept as morphological variables for further analyses. Multivariate regressions were performed between size and morphological variables to test for allometric effects. Multivariate analyses of covariance (MANCOVAs) were then performed to assess the differences between sampled populations. Sex was added as co-factor to control for sexual dimorphism. Size was not added as it was correlated to sampling sites. Hotelling T-squared tests were used for pairwise comparison and the threshold of acceptance of the null hypothesis was divided by the number of pairwise comparisons performed following the Bonferonni correction. Finally, neighbor-joining trees based on Euclidian distances between sampling sites centroids (mean variables values for the individuals in each site) were constructed to visualize the differences between sampling sites.

5) Phylogeographic analyses

The dataset was analyzed under the Bayesian inference and the maximum likelihood criteria. The Bayesian inference was conducted with MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003). MRMODELTEST 2.3 (Nylander 2004) and PAUP* (Swofford 2003) were used to obtain the nucleotide substitution model best fitting the data, according to the AIC criterion (Akaike 1974). Uniform interval priors were selected for the parameters, except for base frequencies, which were assigned a Dirichlet prior (Huelsenbeck and Ronquist 2001). Two independent runs of four incrementally heated Metropolis-coupled MCMC chains were run for 10 million generations. Sampling was done every 1000 generations, yielding 20000 trees. The online version of AWTY (Nylander et al. 2008) was used to assess the convergence of the MCMC chains and to estimate the "burn-in" length. Maximum likelihood searches of the dataset were conducted with RAxML v. 7.0.3 (Stamatakis 2006) using a GTR+Γ+I model and a random starting tree. Nodal support was estimated using 100 bootstrap replicates.

6) Population genetic analyses

The presence of null alleles was assessed with FREENA (Chapuis and Estoup 2007). Sample sites with less than 15 individuals were excluded of the analysis. Mean number of alleles, Shannon's information index, observed heterozygosity, expected heterozygosity, unbiased expected heterozygosity, and fixation Index were assessed over all loci and for each sample site with GENEALEX 6.5 (Peakall and Smouse 2012). Deviation from Hardy-Weinberg equilibrium and linkage disequilibrium between pairs of loci were also tested for each sample site with GENEPOP 4.2.1 (Rousset 2008) using default parameter values.

The Bayesian clustering approach implemented in STRUCTURE 2.3.3 (Pritchard et al. 2000; Falush et al. 2003) was used to describe the genetic structure in the data set. Ten runs were performed for each value of K from 1 to 10 (burn-in period: 50.10^3, 150.10^3 iterations). The admixture model and the assumption of correlated allele frequencies were chosen. The most likely number of clusters (K) was inferred with the mean log-likelihood of the simulations for each value of K and the value of deltaK calculated following (Evanno et al. 2005). Convergence of the MCMC was assessed by checking the stabilization of the parameters α and F.

Results

1) Phylogeography

We obtained sequences for 9 specimens of the native populations, and the sequences of ten additional specimens were retrieved from Genbank. All currently recognized subspecies were represented in this dataset. In addition, we obtained sequences for 39 individuals sampled from introduced populations and a captive stock (North of Paris 5 individuals, South of Paris 5 ind., Heidelberg 9 ind., Barcelona 7 ind., Marseille 2 ind., Alger 2 ind., and captive stock 9 ind.). The output of MRMODELTEST suggested as the best fit the GTR+Γ+I model.

Surprisingly, the sequence from the *P. echo* specimen fall in the clade formed by the *P. krameri* specimens. However, this result was also obtained by Groombridge et al. (2004). Apart from that, the phylogenetic analysis suggests that the African sub-species *P. k. krameri* is paraphyletic and that the Asian subspecies derive from it. The Asian subspecies are unresolved with the portion of cytochrome *b* we used (figure 1). The only *parvirostris* sequence at our disposal was retrived from Genbank and it is included in the clade of the Asian subspecies. The individuals from introduced populations fall in the Asian clade, except the sample from Alger and one of the two samples from Marseille. Some individuals from

introduced populations share identical haplotypes but individuals captured in a single population are never grouped all together (figure 1).

2) Morphology

The two first axes of the PCA performed on log-shaped ratios explain 61.2% of the total variability (34.1 and 27.1% respectively). The centroids of the two Asian subspecies, the two African subspecies and the two populations of Paris are well separated in the morphospace defined by the first two axes of the PCA (figure 2a). In this morphosapce, the centroids of the two populations of Paris appear closer to each other than to the centroids of the other groups but are not superposed. This is also true for the two Asian subspecies and the two African ones. However, the sets of points of all these groups are partially superposed. This pattern is also visible on the neighbor-joining tree calculated with Euclidian distances between the centroid of each population (figure 2a). There is a significant allometric effect (P<2.2e-16), with a positive correlation between size and the first and fifth axes (slope=$1.04.10^{-2}$ and $7.12.10^{-3}$ respectively, SE=$7.01.10^{-4}$ and $1.40.10^{-3}$ respectively) and a negative correlation between size and the second and third axes (slope=$-3.76.10^{-3}$ and $2.26.10^{-3}$ respectively, SE=$7.86.10^{-4}$ and $1.01.10^{-3}$ respectively).The MANCOVA performed on the five first axes showed that the origin of individuals significantly explains their morphology (P<2.2e-16). The pairwise comparisons confirmed that Asian and African subspecies are significantly morphologically different (table 1). There are significant difference between the *P. k. krameri* and *P. k. parvirostris* subspecies, between the *P. k. borealis* and the *P. k. manillensis* subspecies and between the two populations of Paris. Finally, the two populations of Paris are significantly different from the Asian group (table 1).

For the PCA on Procrustes residuals, the first two axes explain 68.2% of the total variability (44.9 and 23.3% respectively). The centroids of the two Asian subspecies, the two African subspecies and the two populations of Paris are disposed in a similar way in the morphospace defined by the first two axes of the PCA performed on log-shaped ratios and Procrustes residuals (figure 2b). The centroid of the population of Barcelona is close to those of Paris. The centroids of the population of Heidelberg and the captive stock are located outside of the region occupied by the other groups in the morphospace, and in opposite sides. The sets of points of all these groups are again partially superposed. This pattern is also visible on the neighbor-joining tree calculated with Euclidian distances between the centroid of each population (figure 2b). There is a significant allometric effect (P<2.2e-16), with a positive correlation between size and the third, fifth and seventh axes (slope=$6.80.10^{-1}$, 1.84

and 2.87 respectively, SE=$3.01.10^{-1}$, $5.06.10^{-1}$ and $6.47.10^{-1}$ respectively) and a negative correlation between size and the first and sixth axes (slope=-1.20 and 2.20 respectively, SE=$1.57.10^{-1}$ and $6.05.10^{-1}$ respectively). The MANCOVA performed on the seven first axes showed that the origin of individuals significantly explains their morphology (P<2.2e-16). The pairwise comparisons confirmed that Asian and African subspecies are significantly morphologically different (table 1). However, this time there are no significant differences between the *P. k. krameri* and *P. k. parvirostris* subspecies, and between the *P. k. borealis* and the *P. k. manillensis* subspecies. All the introduced populations were significantly different from each other and there were also significantly different from the Asian group (table 1).

3) Population genetics

Amplification of the microsatellite loci was successful in the majority of samples (98.3% of loci successfully amplified over all samples) and all loci were polymorphic. No null alleles were detected. The genetic diversity ranged from 0.71 to 0.81 (table S4). The populations sampled did not significantly deviate from Hardy-Weinberg equilibrium (HWE) nor presented linkage disequilibrium, except for Heidelberg and Barcelona. Indeed, for these populations, 39% of the loci deviated from HWE, and 58 and 75% of the pairs of loci showed linkage disequilibrium respectively (table S4).

The log-likelihood of the simulations run with STRUCTURE increases sharply until K=5 and then more slowly to reach a plateau (figure S5). The delta K first peak for K=2 but there is a second more important peak at K=5 (figure S6). For K= 2, there are two situations: either the individuals of the South of Paris are separated from all the others, or it is those from Spain and Germany. For K=3, the individuals from respectively the South of Paris, Spain and Germany are in two separated clusters and the last cluster is composed of the individuals from the North of Paris, Marseille, Alger and captivity. For K=4, the different runs provide different dispatching of individuals into the four clusters. For K= 5, all the runs give the same clustering of individuals. Each population is well distinguished except for the individuals of Marseille and Alger which are mainly grouped with those of the North of Paris. Twelve individuals of the North of Paris have however a mixed origin between the clusters of the North and South of Paris. Five of them even have more than 95% of their origin assigned to the cluster of the South of Paris. This indicates that these two populations are not isolated. After K=5, no supplementary clusters ae defined.

Discussion

1) Role of phylogenetic origin in morphological differences

We found that the introduced populations of Paris, Barcelona and Heidelberg are related to the Asian clade, indicating that they have a common origin. The Asian subspecies are not resolved in the phylogenetic tree we obtained from the cytochrome *b* gene. This might be caused by a lack of resolution due to the fact that we used only a single mitochondrial gene. However, the possibility that the two morphological subspecies are not genetically differentiated cannot be excluded. The morphometric analyses on classical measurements and beak shape both show that there are significant morphological differences between all the introduced populations we studied. As these populations have a common origin, their phylogenetic origin cannot explain these differences.

2) Role of recent demographic history in morphological differences

Individuals from populations in different environments were significantly morphologically different. This could indicate that the Ring-necked parakeets are adapted to the different environments in which they were introduced. However, there were also significant differences in both morphological analyses between the two populations established in similar environments near Paris. It is thus improbable that local adaptation to climatic conditions is the cause of the morphological differentiation observed, at least in these two populations. The study of additional populations introduced in environments similar to Barcelona and Heidelberg would however be necessary to assess if the morphological changes found in these populations are likely to be adaptive or not.

The population genetics analysis shows that there are differences in neutral genetic loci between the different introduced populations studied. This indicates that recent demographic processes such as founder effects and genetic drift have resulted in stochastic evolution in these populations. The morphological differences existing between introduced populations correspond to the genetic differences observed with neutral genetic loci. This suggests that the cause of the morphological and genetic differentiations observed is the same. Stochastic evolution caused by recent demographic processes is thus a more probable explanation to the phenotypic differences observed than rapid adaptation.

Interestingly, the individuals from captivity were morphologically very different from all the other individuals. This could be caused by the fact that the pictures we used were taken by a different person but as they were taken in standardized conditions, like for individuals of Barcelona that were found to be morphologically close to the individuals of Paris, this seems

improbable. Moreover, they were also genetically different than the individuals from the other populations. Stochastic demographic processes are thus also the probable cause of this morphological difference. If a part of the individuals introduced in Europe were not directly introduced but come from captive breeding, it is probable that they went through multiple founder events and bottlenecks. This could explain the strong genetic structuration observed between populations (Clegg et al. 2002). Under this hypothesis, the important morphological differences observed between the individuals of Heidelberg and those of the other introduced populations could be explained by the fact at least two foundation events have occurred in this population as it was founded in 1990 from a close by population itself founded in 1974.

3) Other possible explanations for the invasive success of Ring-necked parakeets

Although rapid morphological adaptation does not seem a probable explanation for the establishment success of the Ring-necked parakeet in Europe, selection on other phenotypic traits might have favored this establishment. Indeed, physiological or behavioral adaptations could also facilitate the establishment in environments with different climatic conditions. The study of these traits is thus also necessary to assess if rapid adaptation occurred in introduced populations.

Another possible explanation for the establishment of Ring-necked parakeet in temperate regions is that they could already be adapted to these climatic conditions. Indeed, we have showed that most of the introduced individuals we studied have an Asian origin although similar proportions of individuals from Asia and Africa have been imported in Europe (CITES 2013, table S1). Only one individual from Marseille and one from Alger are grouped with specimens of the *P. k. krameri* subspecies. This might be because the native range of the Asian subspecies covers a wider climatic range than the range of the African subspecies. Indeed, in Asia, the subspecies *P. k. borealis* is found in the Himalayan region up to the altitude of 1600 m (Parr and Juniper 2010). This subspecies might thus be adapted to climatic conditions matching those found in Europe, contrary to the African subspecies which might only be able to survive in Mediterranean regions. The fact that we did not find any individuals of Barcelona with an African origin however indicates that they might not be able to reproduce there. A study on captive Ring-necked parakeets confirm the hypothesis that they are pre-adapted to tolerate cold climate (Thabethe et al. 2013). Indeed, it showed that they have seasonal thermoregulatory response that allow them to save energy and that they are not in hypothermia at 5°C. However, the Ring-necked parakeet occurrence in Europe was found to be negatively correlated with the number of frost days (Strubbe and Matthysen

2009a). Moreover, in the United Kingdom the hatching success is reduced in comparison to that in the native range of parakeets (Shwartz et al. 2009). This suggests that the environmental conditions in the introduced range are not optimal for the species.

Another trait that could explain the success of the Ring-necked parakeet in Europe is its opportunistic behavior which makes it a good urban exploiter. Indeed, behavioral flexibility has been positively correlated to invasive success in a study on 69 invasive bird species (Sol et al. 2002). Ring-necked parakeets have been observed to feed on a wide range of food sources in introduced populations (Clergeau et al. 2009; Clergeau and Vergnes 2011) as well as in their native range (Ahmad et al. 2011). This generalist character might help them to forage efficiently even in new environments and thus resist to cold temperatures. Indeed, they find food sources all year long in suburban areas thanks to the diversity of trees and shrubs. In addition, a large part of their diet is composed of highly nutritious seeds that they get from bird feeders (Clergeau and Vergnes 2011). This rich diet might thus also help them to go through the cold season. Ring-necked parakeets also demonstrated plasticity in their reproductive behaviors as they were found nesting in nest boxes but also in thermal insulation on buildings where they enlarge holes made by other birds (Braun and Wink 2013). This capacity to exploit suburban environments might explain why they manage to establish in environments with different climatic conditions. This might also explain why they have not yet expanded in more rural areas.

Conclusion

This study shows that although there are significant morphological differences between introduced populations and their source, and between populations introduced in different environments, these differences are more likely to result from stochastic evolution than from rapid adaptation. However, the hypothesis of rapid adaptation cannot be completely excluded and studies of additional populations in similar climatic regions are necessary to investigate further this hypothesis. The study of other phenotypic traits such as behavior and physiology is also necessary to assess if rapid adaptation occurred in introduced populations. Another interesting finding of this study is that the populations of the North and the South of Paris are not isolated. This could have implications for their management as they must be considered as connected units. Finally, we show that Ring-necked parakeets found in the wild in Europe have an Asian origin in the majority of cases. This is the first time this information is confirmed with genetic data to our knowledge. Assessing why individuals of African origin

are underrepresented in invasive populations could help to understand the factors which have favored the establishment of the Ring-necked parakeet in Europe.

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Tables

Table 1: Results of the pairwise comparisons between subspecies / introduced populations conducted with Hotelling's T-squared tests. df: degrees of freedom, nx and ny: sample sizes for the two populations compared.

Populations compared	Hotelling's T^2 statistic	scaling factor	df	nx	ny	P-value
Log-shaped ratios						
krameri/parvirostris	17.48	0.19	5; 113	105	14	7.02e-03*
borealis/manillensis	40.89	0.20	5; 186	129	63	7.41e-07*
Paris.N/Paris.S	30.40	0.19	5; 83	43	46	1.21e-04*
Africa/Asia	570.08	0.20	5; 305	119	192	0.00*
Asia/Paris.N	115.84	0.20	5; 229	192	43	0.00*
Asia/Paris.S	160.85	0.20	5; 232	192	46	0.00*
Procrustes residuals						
krameri/parvirostris	15.07	0.14	7; 127	118	17	5.30e-02
borealis/manillensis	8.16	0.14	7; 170	114	64	0.35
Africa/Asia	606.36	0.14	7; 305	135	178	0.00*
Asia/Paris.N	63.17	0.14	7; 211	178	41	1.85e-09*
Asia/Paris.S	57.50	0.14	7; 223	178	53	1.15e-08*
Asia/Heidelberg	382.96	0.14	7; 203	178	33	0.00*
Asia/Barcelona	30.05	0.14	7; 180	178	10	2.91e-04*
Asia/Captivity	144.29	0.14	7; 184	178	14	0.00*
Paris.N/Paris.S	32.31	0.13	7; 86	41	53	3.91e-04*
Paris/Heidelberg	144.86	0.14	7; 119	94	33	0.00*
Paris/Barcelona	48.54	0.13	7; 96	94	10	2.65e-06*
Paris/Captivity	186.68	0.13	7; 100	94	14	0.00*
Heidelberg/Barcelona	86.52	0.12	7; 35	33	10	4.70e-07*
Heidelberg/Captivity	212.87	0.12	7; 39	33	14	6.53e-13*
Barcelona/Captivity	58.17	0.10	7; 16	10	14	1.42e-03*

*Indicates a significant difference between populations compared. The significance threshold of 0.05 was divided by six for comparisons on log-shaped ratios and by 15 for comparisons on Procrustes residuals to account for multiple testing following the Bonferroni correction.

Figures

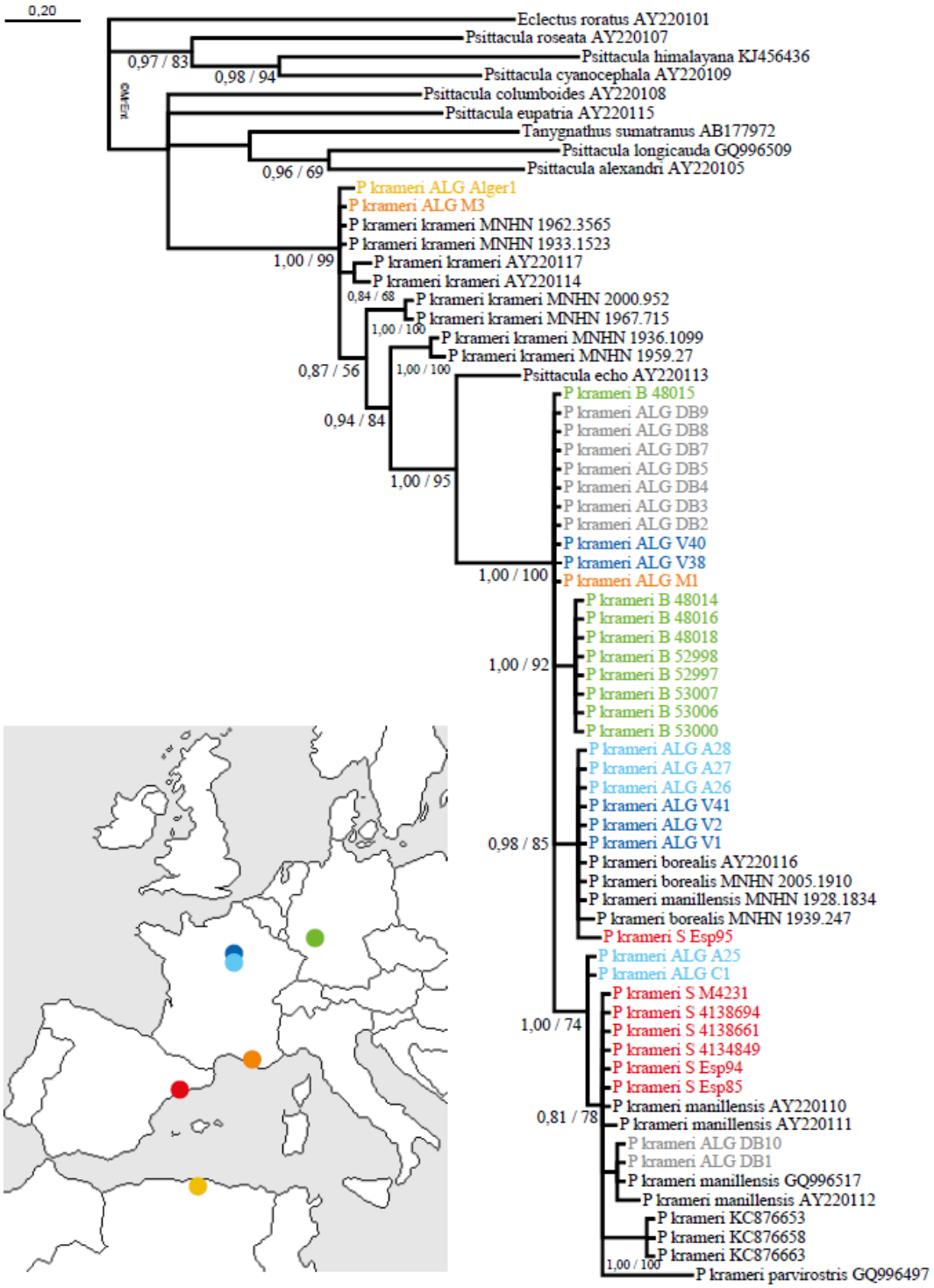

Fig. 1: The majority rule consensus tree obtained from the Bayesian analysis of the cytochrome b gene. The support values indicated at the node are the posterior probability (threshold 0.50) and the bootstrap support (threshold 50%) obtained from the maximum likelihood analysis, respectively. Colors refer to the populations represented on the map where individuals were sampled. Individuals in grey come from captivity, and individuals in black are specimens from the native range.

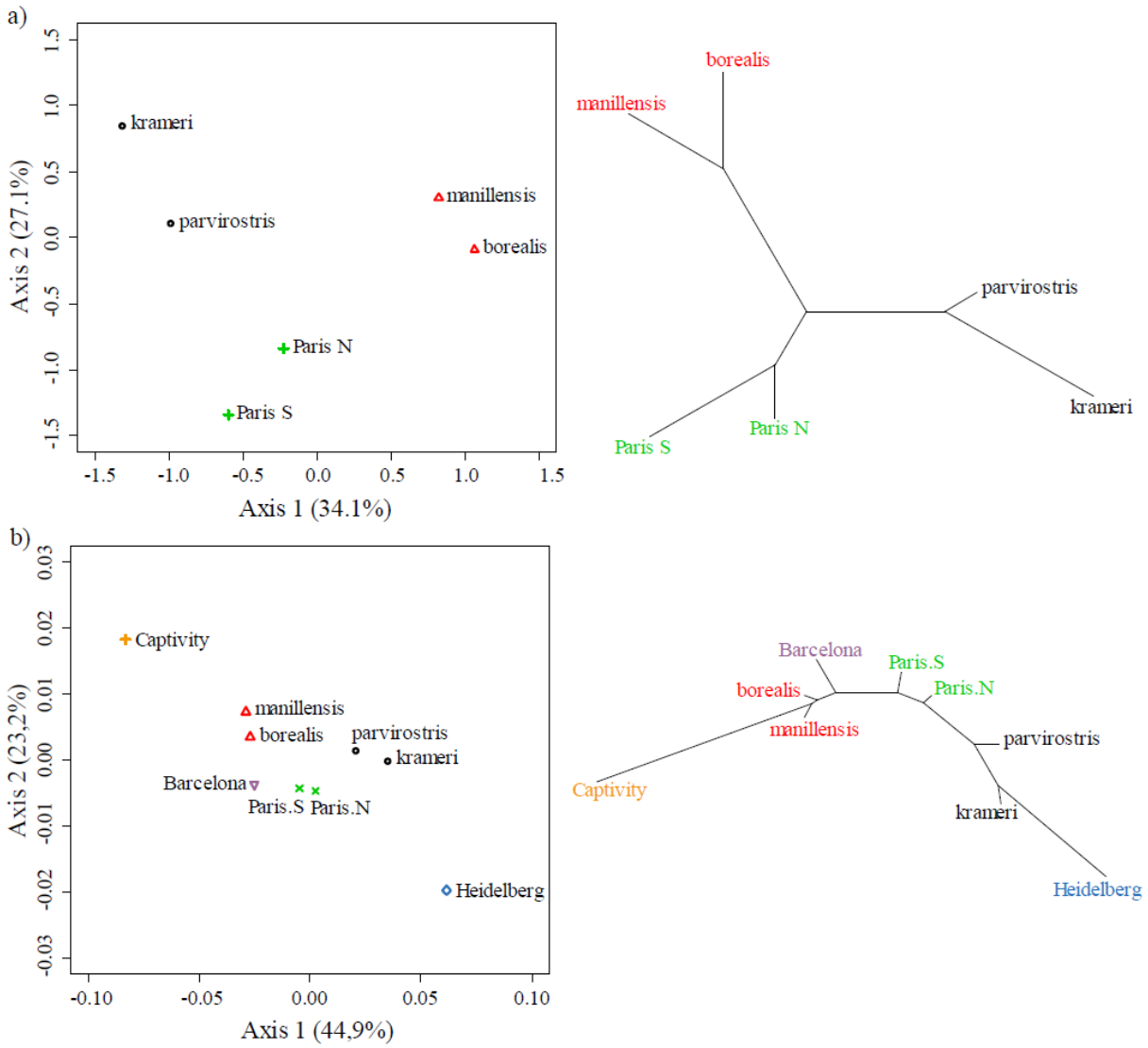

Fig. 2: Plots of the centroid of each subspecies and introduced population studied on the two first axes of the PCA done on the log-shaped ratios (a) or on Procrustes residuals (b) and neighbor-joining trees calculated with the Euclidian distances between centroids coordinates on the five first axes of the PCA done on the log-shaped ratios (a) or on the seven first axes of the PCA done on the Procrustes residuals. Correlation circles and conformations associated to the axis of the PCAs are in the supplementary materials (figures S3 and S4 respectively).

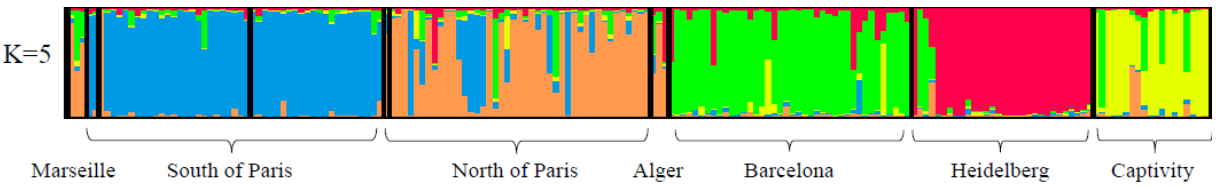

Fig. 3: Cluster assignments of individuals obtained with STRUCTURE K=5. The results of the 10 runs were pooled together using CLUMPP (Jakobsson and Rosenberg 2007). Each vertical line represents a single individual and individuals are grouped by sampling site.

Supplementary Material

1) Amplification Protocols

Cytochrome b:

Reagent quantities for amplification in a single fragment: 13.94µL of Milli-Q water, 2µL of polymerase buffer 10x (Qiagen), 1.5µL of $MgCl_2$ (Qiagen, 25mM), 1µL of Dimethyl sulfoxide (DMSO), 0.8µL of dNTPs mix (1.7mM each), 0.32µL of each primer (10µM), 0.12µL of DNA polymerase (Qiagen, Taq 5 units/µL). Cycling conditions: 94°C, 5 min.; (94°C, 40 sec.; 57°C, 40 sec.; 72°C, 60sec.) x 40 cycles; 72°C, 5 min.

For the amplification of short fragments, illustra™ Hot Start Mixes (GE Healthcare) were used with the following reagent quantities: 19µl of Milli-Q water, 1µL of $MgCl_2$ (Qiagen, 25mM), 1µL of each primer (10µM). Cycling conditions: 94°C, 5 min.; (94°C, 40 sec.; 61°C, 40 sec.; 72°C, 60sec.) x 4 cycles; (94°C, 40 sec.; 59°C, 40 sec.; 72°C, 60sec.) x 4 cycles; (94°C, 40 sec.; 57°C, 40 sec.; 72°C, 60sec.) x 32 cycles; 72°C, 5 min.

Microsatellites loci:

Microsatellite loci were amplified in three multiplex and tagged with fluorescent forward primers (dyes: 6-FAM, VIC, NED, PET; Applied Biosystems). PCR amplifications were done using the following reagent quantities: 1.25µL of the primer mix (1µM of each primer and TE buffer), 4µL of RNase-free water (Qiagen), 6.25µL of 2x Type-it Multiplex PCR Master Mix (Qiagen) in a final volume of 11.5µL. Cycling conditions: 95°C, 5 min.; (95°C, 30 sec.; 57°C, 90 sec.; 72°C, 30sec.) x 25 cycles; 60°C, 30 min.

2) Tables

Table S1: Number of Ring-necked parakeets imported in European countries from Asia and Africa and between 1985 and 2010 according to the CITES data base.

Importing country	Importations from Africa Counts and proportion of total	Importations from Asia Counts and proportion of total	Total
Italy	27 955 - 29%	69 766 - 71%	97 721
Portugal	41 087 - 85%	7 010 - 15%	20 522
Spain	27 007 - 64%	15 250 - 36%	42 257
UK	15 278 - 65%	8 145 - 35%	23 423
France	17 568 - 86%	2 974 - 14%	20 522
Germany	13 211 - 67%	6 597 - 33%	19 808
Belgium	6 213 - 65%	3 379 - 35%	9 592
Netherlands	4 815 - 50%	4470 - 50%	9585

Table S2: details of the specimens used in the phylogeographic analysis.

Species	Subspecies	Country	Locality	ID Number	Genbank
P. krameri	*manillensis*	Sri Lanka	Ratnapura	MNHN 1928.1834	Unpub ALG
P. krameri	*manillensis*	NA	NA	NA	GQ996517
P. krameri	*manillensis*	NA	NA	NA	AY220112
P. krameri	*manillensis*	NA	NA	NA	AY220111
P. krameri	*manillensis*	NA	NA	NA	AY220110
P. krameri	*borealis*	India	Madhya Pradesh - Mandla	MNHN 1939.247	Unpub ALG
P. krameri	*borealis*	India	Madhya Pradesh	MNHN 2005.1910	Unpub ALG
P. krameri	*borealis*	Pakistan	NA	NA	KC876658
P. krameri	*borealis*	Pakistan	NA	NA	KC876653
P. krameri	*borealis*	Pakistan	NA	NA	KC876663
P. krameri	*borealis*	NA	NA	NA	AY220116
P. krameri	*parvirostris*	NA	NA	BNHM 1927.5.3.1	GQ996497
P. krameri	*krameri*	NA	NA	NA	AY220114
P. krameri	*krameri*	NA	NA	NA	AY220117
P. krameri	*krameri*	Mali	Bandiagara - Gouandaka	MNHN 1933.1523	Unpub ALG
P. krameri	*krameri*	Mali	Koulikoro - Koulikoro	MNHN 1962.3565	Unpub ALG
P. krameri	*krameri*	Tchad	Baguirmi - East of Ndjamena	MNHN 1936.1099	Unpub ALG
P. krameri	*krameri*	Tchad	West Ennedi	MNHN 1959.27	Unpub ALG
P. krameri	*krameri*	Cameroun	Logone-et-Chari - Waza	MNHN 1967.715	Unpub ALG
P. krameri	*krameri*	Cameroun	NA	MNHN 2000.952	Unpub ALG
P. krameri	NA	Algeria	Alger	ALG Ager1	Unpub ALG
P. krameri	NA	France	Marseille	ALG M1	Unpub ALG
P. krameri	NA	France	Marseille	ALG M3	Unpub ALG
P. krameri	NA	France	Villepinte	ALG V1	Unpub ALG
P. krameri	NA	France	Villepinte	ALG V2	Unpub ALG

P. krameri	NA	France	Villepinte	ALG V38	Unpub ALG
P. krameri	NA	France	Villepinte	ALG V40	Unpub ALG
P. krameri	NA	France	Villepinte	ALG V41	Unpub ALG
P. krameri	NA	France	Antony	ALG A25	Unpub ALG
P. krameri	NA	France	Antony	ALG A26	Unpub ALG
P. krameri	NA	France	Antony	ALG A27	Unpub ALG
P. krameri	NA	France	Antony	ALG A28	Unpub ALG
P. krameri	NA	France	Chatenay-Malabry	ALG C1	Unpub ALG
P. krameri	NA	France	Captivity	ALG DB1	Unpub ALG
P. krameri	NA	France	Captivity	ALG DB2	Unpub ALG
P. krameri	NA	France	Captivity	ALG DB3	Unpub ALG
P. krameri	NA	France	Captivity	ALG DB4	Unpub ALG
P. krameri	NA	France	Captivity	ALG DB5	Unpub ALG
P. krameri	NA	France	Captivity	ALG DB7	Unpub ALG
P. krameri	NA	France	Captivity	ALG DB8	Unpub ALG
P. krameri	NA	France	Captivity	ALG DB9	Unpub ALG
P. krameri	NA	France	Captivity	ALG DB10	Unpub ALG
P. krameri	NA	Germany	Heidelberg	B 48014	Unpub ALG
P. krameri	NA	Germany	Heidelberg	B 48015	Unpub ALG
P. krameri	NA	Germany	Heidelberg	B 48016	Unpub ALG
P. krameri	NA	Germany	Heidelberg	B 48018	Unpub ALG
P. krameri	NA	Germany	Heidelberg	B 52997	Unpub ALG
P. krameri	NA	Germany	Heidelberg	B 52998	Unpub ALG
P. krameri	NA	Germany	Heidelberg	B 53000	Unpub ALG
P. krameri	NA	Germany	Heidelberg	B 53006	Unpub ALG
P. krameri	NA	Germany	Heidelberg	B 53007	Unpub ALG
P. krameri	NA	Spain	Barcelona	S Esp95	Unpub ALG
P. krameri	NA	Spain	Barcelona	S M4231	Unpub ALG
P. krameri	NA	Spain	Barcelona	S 4138695	Unpub ALG
P. krameri	NA	Spain	Barcelona	S M4234	Unpub ALG
P. krameri	NA	Spain	Barcelona	S 4134849	Unpub ALG
P. krameri	NA	Spain	Barcelona	S 4138661	Unpub ALG
P. krameri	NA	Spain	Barcelona	S 4138694	Unpub ALG
P. echo	NA	NA	NA	NA	AY220113
P. columboides	NA	NA	NA	NA	AY220108
P. eupatria	NA	NA	NA	NA	AY220115
P. longicauda	NA	NA	NA	NA	GQ996509
P. Alexandri	NA	NA	NA	NA	AY220105
P. cyanocephala	NA	NA	NA	NA	AY220109
P. himalayana	NA	NA	NA	NA	KJ456436
P. roseata	NA	NA	NA	NA	AY220107
Tanygnathus sumatranus	NA	NA	NA	NA	AB177972
Eclectus roratus	NA	NA	NA	NA	AY220101

Table S3: Primers used to amplify fragments of the cytochrome *b* gene.

Gene	Primer F		Primer R	
Cytb (single fragment)	ALG-L14841	CCATCCAACATCTCAGCATGATGAAA	ALG-cytb-R813	GAATAGGTTGGCGGCGAGTGTTCAGA
Cytb (1st fragment)	ALG-L14841	CCATCCAACATCTCAGCATGATGAAA	ALG-cytb-R221	GCCTCATGGTAAGACATAGCCAACGA
Cytb (2nd fragment)	ALG-cytb-F173	CCTGAAACACAGGAATCATCCTCCTA	ALG-cytb-R422	TGATTCGTGGAGAAAGGTTAGGTGGA
Cytb (3rd fragment)	ALG-cytb-F356	CCACCTTAACACGATTCTTCGCCCTA	ALG-cytb-R605	AGTTGTTAGGGGGTTTGCTGGGGTGA
Cytb (4th fragment)	ALG-cytb-F551	CCCTCACCACCCTTGCCCTATTCTCA	ALG-cytb-R813	GAATAGGTTGGCGGCGAGTGTTCAGA

Table S4: Mean number of individuals (N), number of alleles (Na), Shannon's diversity Index (I), observed heterozygosity (Ho), expected heterozygosity (He), unbiased expected heterozygosity (uHe), fixation index (F) per sampling site and over all loci. Proportion of loci deviating from Hardy-Weinberg equilibrium (HWE) and proportion of pair of loci showing linkage disequilibrium (LD) per sampling site.

Population		**N**	**Na**	**Shannon I**	**Ho**	**He**	**uHe**	**F**	**HWE**	**LD**
Antony	**Mean**	23.556	7.278	1.630	0.684	0.755	0.771	0.088	4/18	1/153
	SE	0.459	0.547	0.079	0.023	0.020	0.020	0.033		
Chatenay	**Mean**	21.944	7.000	1.570	0.750	0.734	0.751	-0.020	2/18	6/153
	SE	0.056	0.560	0.081	0.032	0.021	0.022	0.031		
Villepinte	**Mean**	42.111	8.833	1.759	0.750	0.776	0.785	0.027	3/18	26/153
	SE	0.435	0.701	0.080	0.015	0.019	0.019	0.020		
Barcelona	**Mean**	39.778	10.278	1.893	0.790	0.798	0.809	0.005	7/18	115/153
	SE	0.129	1.038	0.105	0.030	0.020	0.021	0.038		
Heidelberg	**Mean**	29.722	7.944	1.648	0.760	0.745	0.757	-0.018	7/18	89/153
	SE	0.109	0.602	0.082	0.030	0.023	0.024	0.018		
Captivity	**Mean**	18.944	6.833	1.498	0.662	0.691	0.709	0.026	4/18	16/153
	SE	0.056	0.579	0.114	0.041	0.042	0.044	0.036		

3) Figures

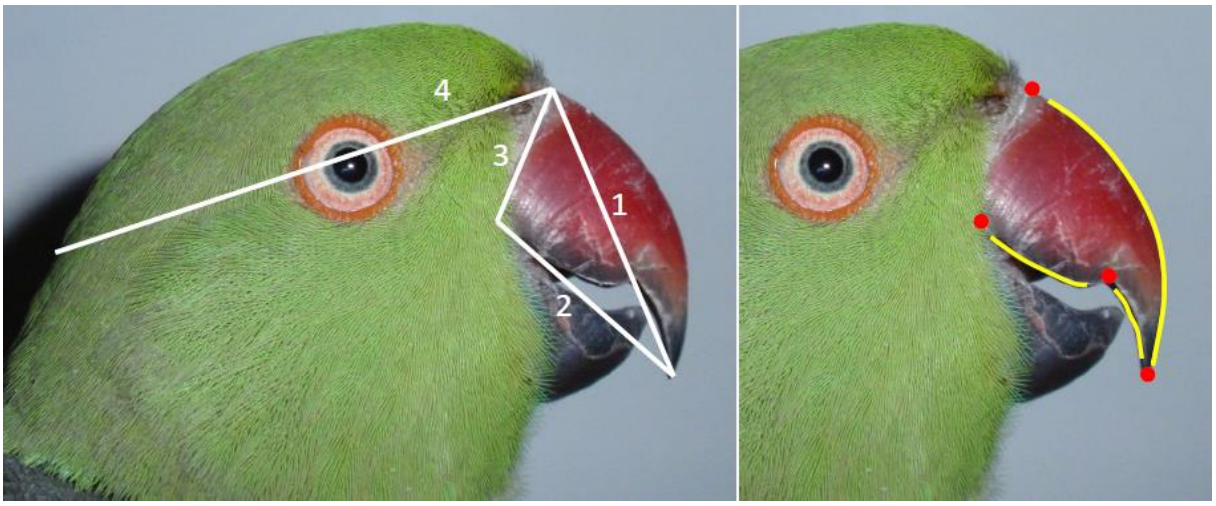

Fig. S1: Left: Measurements taken on the beak of parakeets; 1: upper mandible length (n°1), 2: upper mandible length (n°2), 3: upper mandible depth, and 4: cranium length. Right: locations of the four landmarks and 3 outline curves (21 semi-lankmarks) digitized on pictures of the beak in lateral view.

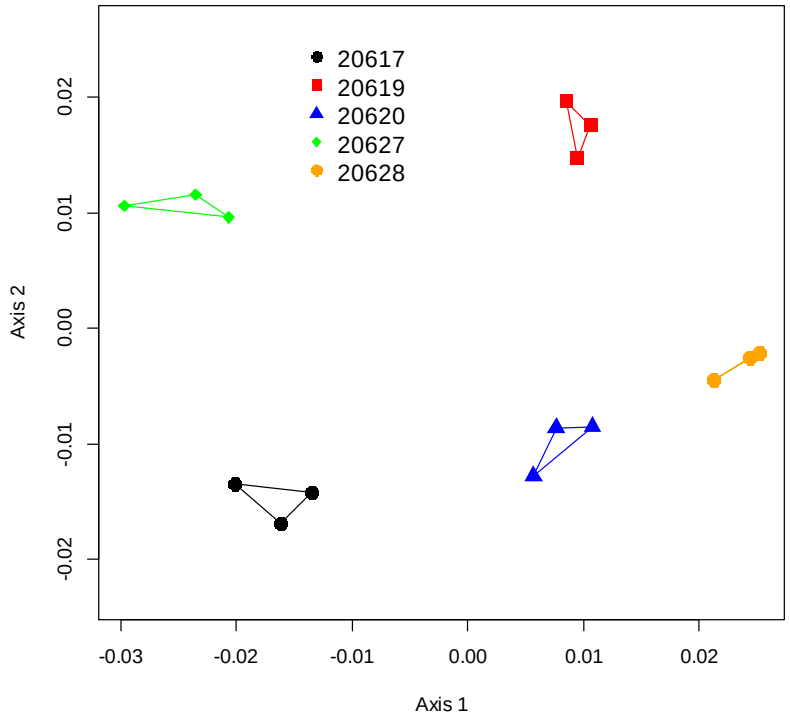

Fig. S2: PCA performed on beak conformation to test the repeatability of the digitization process. Each color represents an individual. Three repetitions where done for each of them.

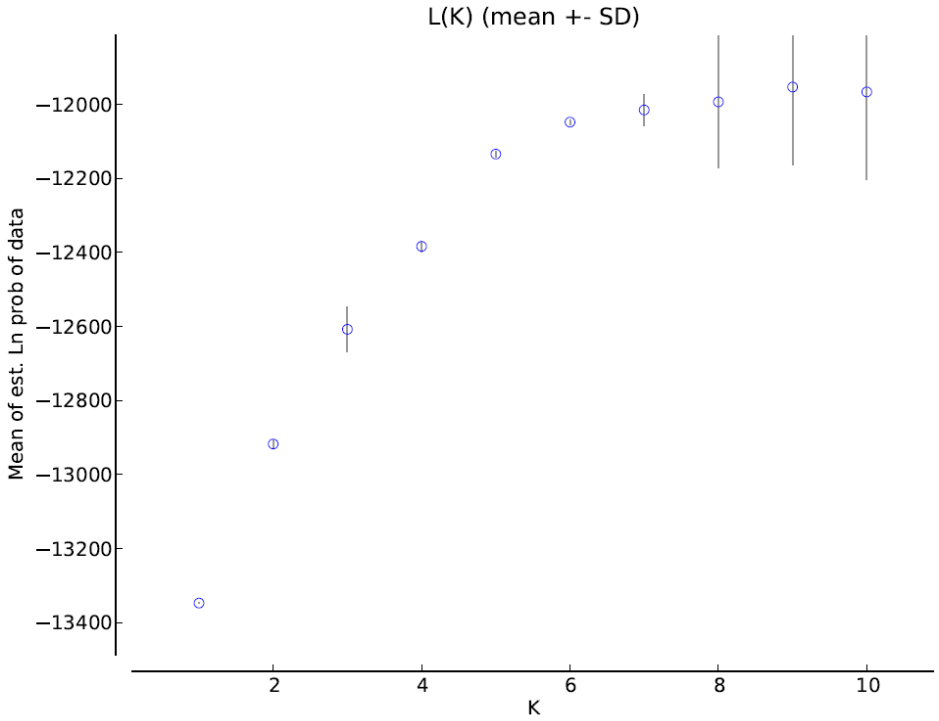

Fig. S3: mean log-likelihood of the 10 simulations run with STRUCTURE (Pritchard et al. 2000; Falush et al. 2003) for each value of K and calculated with STRUCTURE HARVESTER (Earl et al. 2012)

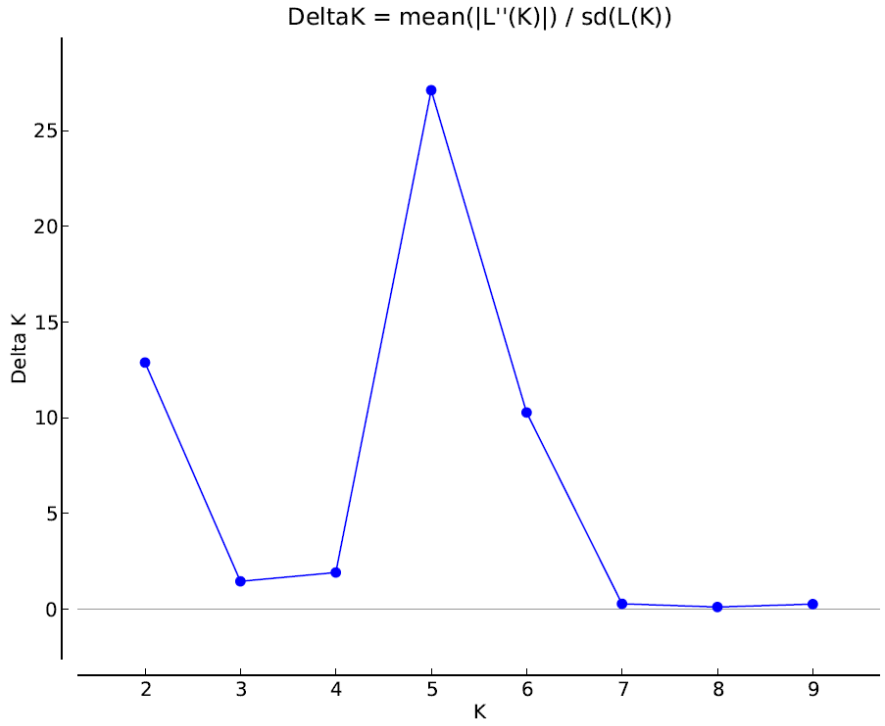

Fig. S4: Delta K for the 10 simulations run with STRUCTURE (Pritchard et al. 2000; Falush et al. 2003) for each value of K and calculated with STRUCTURE HARVESTER (Earl et al. 2012) following (Evanno et al. 2005).

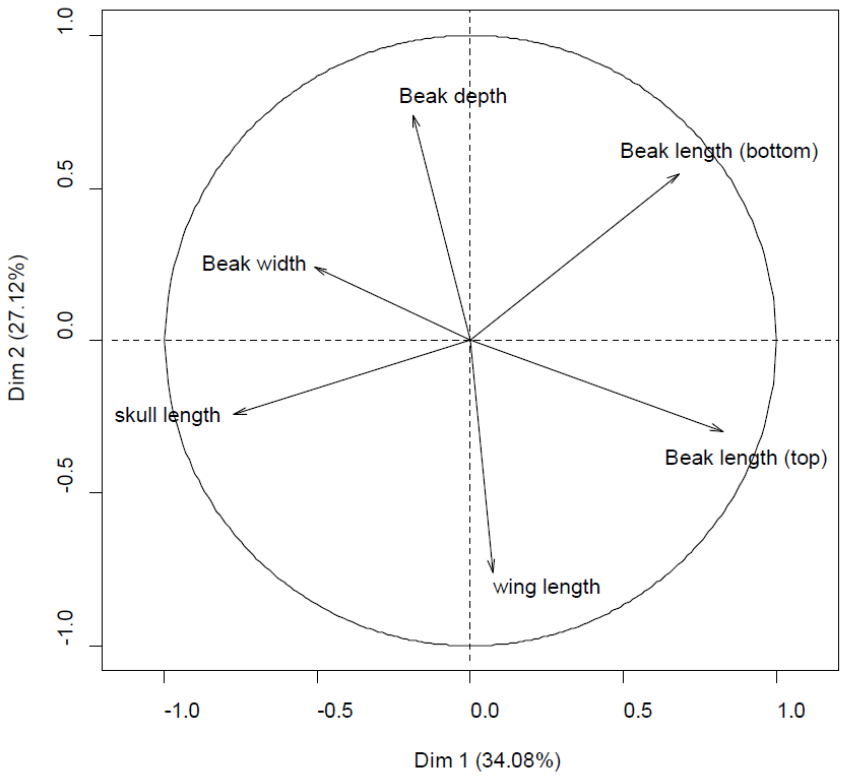

Fig. S5: Correlation circles for the plane defined by the first two axes of the PCA on log-shpaed ratios. B.LH: first beak length; B.LB: second beak length; B.E: beak width; B.H: beak depth; aile: folded wing length; crane: cranium length.

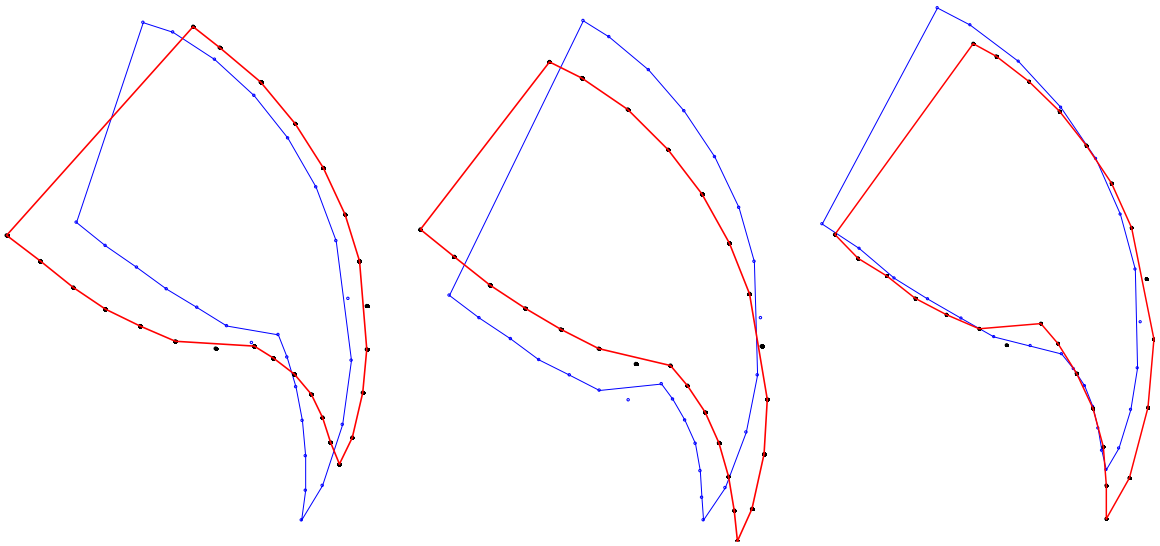

Fig. S6: Beak conformation associated to the positive (red) and negative (blue) extremities of axes 1 (left), 2 (middle) and 3 (right) of the PCA on Procrustes residuals.

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IV. Discussion

IV.1. Advances made

IV.1.1. Beware of correlations between phenotypic and environmental changes

The study of morphology in both species showed that there are significant morphological differences between the individuals of each introduced populations and those of the native range. This indicates that morphological changes have occurred in populations since their introduction. Moreover, in the morphospaces we defined for each species, the introduced populations are approximately grouped in the same region. The morphological changes that occurred in introduced populations have therefore been more or less the same for each population (figure 17). Without further analyses, these correlations between a sharp environmental change (i.e. the introduction in islands for a continental species or in a temperate region for a tropical species) and morphological changes occurring more or less in a similar way in several populations could have been interpreted as the result of rapid adaptation to new the new environment.

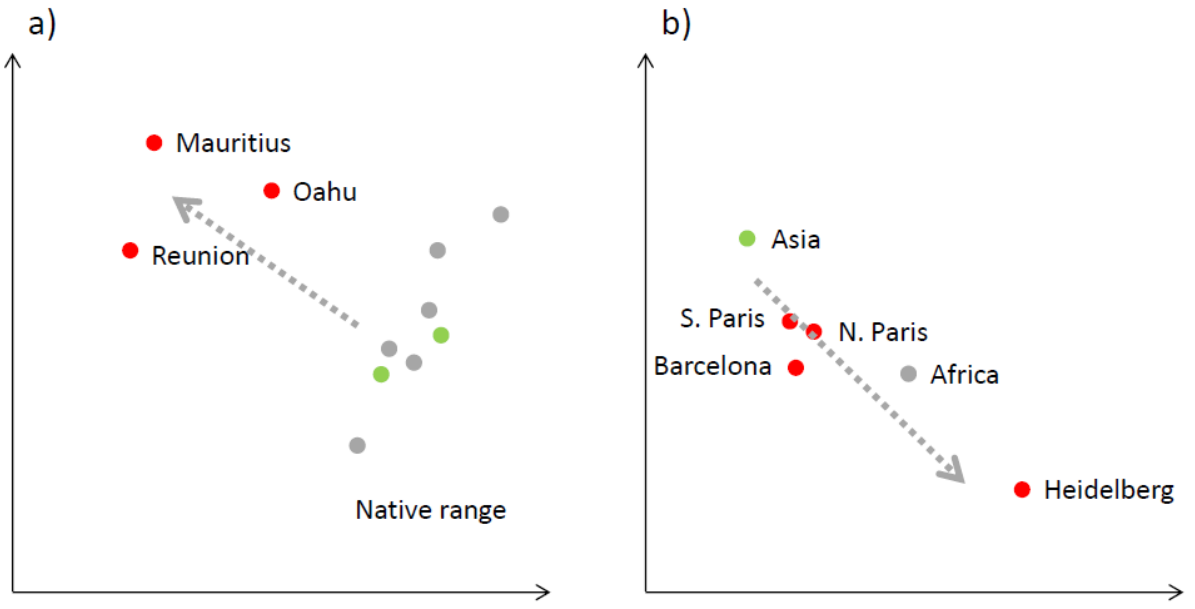

Fig. 17: schematic direction of morphological differentiation in introduced populations of a) Red-whiskered bulbuls and b) Ring-necked parakeets. Points correspond to the centroid of each population and are represented schematically in the morphospaces defined with the first two axes of the PCA for both species. Green indicates the source populations, grey indicates other populations in the native range, red indicates introduced populations and the arrow indicates the direction of the morphological differentiation.

In this thesis, we tested alternative hypotheses to explain these morphological differentiations. We showed that, in seven out of the eight introduced populations studied, stochastic evolution resulting from recent demographic history was a possible cause for the morphological differentiations we observed. The only exception was the case of Red-whiskered bulbuls of Mauritius. Indeed, in this island, the morphological differences existing between the two coasts cannot be explained by a difference in phylogenetic origin or demographic mechanisms. In this case, local adaptation or phenotypic plasticity could explain the morphological differences observed.

With this work, we thus highlighted the importance of stochastic demographic processes in explaining population differentiation in invasive species. We cannot exclude that local adaptation or phenotypic plasticity are also driving forces for the morphological patterns we observe. In fact, it is even probable that all these mechanisms act at the same time on morphological features. However, our results show that there is a need to assess the role of stochastic evolution in studies on rapid phenotypic changes before concluding that they are caused by rapid adaptation. This is especially true when knowing if a change is adaptive will have direct ecological implications, for example in the case of invasive or endangered species. In the studies on rapid adaptation listed in the introduction, there might thus be cases in which rapid adaptation did not occur after all, especially in studies on invasive species as they are more susceptible to have gone through stochastic evolution than other populations.

IV.1.2. What about rapid adaptation and phenotypic plasticity?

Among the eight introduced populations studied during my thesis, we showed that historical factors were likely explanations for the morphological differentiations we observed. However, we cannot exclude that rapid adaptation and phenotypic plasticity are also acting in shaping these morphological patterns.

The differences between replicated environments could be explicated by the fact that introduced populations are in the process of adapting to local conditions, but that because of stochastic demographic processes, they are evolving from different starting points (*i.e.* different pools of genotypes). Indeed, founder effects, bottlenecks and admixture following introduction events can have a strong impact on the genetic diversity of a population (Sakai *et al.* 2001; Facon *et al.* 2008). Moreover, standing genetic diversity is assumed to be an important component in rapid adaptation (Barrett & Schluter 2008). The populations of

'replicate' environments might therefore be in the process of reaching a common optimum but are following different routes in the adaptive landscape because they had different starting points. In addition, the adaptive landscape can be visualized as a physical landscape where the elevation of each point represents the fitness of an associated genotype (Wright 1932). This landscape can present several peaks that correspond to several local fitness maxima. If there are several local maxima in the adaptive landscape of a species, some populations under selection might reach the top of one peak while others might reach the top of other peaks. In this case, the populations can be trapped in local maxima as it is unlikely that they will go down the peak under unchanged selective pressures. It would thus be possible that the different 'replicate' populations we studied are trapped in different local maxima because they started from different points in the adaptive landscape. This could explain the morphological differences observed between replicated environments. In this case, the comparative approach we used in this thesis is not sufficient to say if the phenotypes observed in introduced populations are adaptive or not. Moreover, it is possible that the environments we considered as "replicate" are in fact quite different for our study species. In this case, they might have got locally adapted to these different environments which would explain the morphological differences observed. It would thus be interesting to collect ecological data on the different environments we study to have a better idea of their degree of similarity.

In addition to this, selection on other traits than those we have studied might have played a role in the establishment of the introduced populations we studied. Indeed, changes in physiology, behavior or life history traits can also favor the establishment of a species introduced in a new environment. For example, physiological adaptations such as a modified metabolic rate or seasonal accumulation of fat can enable to cope with cold climates (Thabethe *et al.* 2013). Changes in feeding behavior (Yonekura *et al.* 2007), migratory behavior (Able & Belthoff 1998; Evans *et al.* 2012), anti-predator behavior (Anson & Dickman 2013) and life history traits (Bøhn *et al.* 2004; Amundsen *et al.* 2012) have been reported upon arrival in a new environment and are suspected to be adaptive. Thus, the study of other traits in our study populations would be necessary to know if selection has acted on these traits after their introduction.

Finally, it is possible that the morphological differentiations we observed are caused partially by phenotypic plasticity. Indeed, although morphological features are believed to be quite heritable in birds, there is still room for environmental effects. Estimating the heritability of the traits we studied would thus be interesting to assess the role of phenotypic plasticity in the morphological differentiations we observed.

IV.2. Perspectives

IV.2.1. Improving sampling of introduced populations

A first improvement that could be made is to increase the number of sample sites for both species. For Ring-necked parakeets, finding replicates of the Mediterranean and the semi-continental populations would allow assessing if the morphotypes we identified there are adaptive or not. The populations of Roma (Italy) and Worms (Germany) would be good candidates as they were founded at the same period as the populations we studied (Braun 2009), and are in the good climatic regions. It would also be interesting to sample the population of Neckarhausen as it is the supposed source of the population of Heidelberg (Braun 2009). We could thus assess the effect of successive foundation events on phenotypic differentiation. For Red-whiskered bulbuls, getting data from populations in other kinds of environments than islands would enable us to see if rapid adaptation happened there or if we only see a phenomenon linked to island populations. The populations of Florida and California could be used, provided that they have the same source as the populations we studied until now.

We could also include other species that are susceptible to have been through rapid adaptation to increase the chances of finding cases of rapid adaptation. We could for example chose the Red-vented bulbul (*Pycnonotus cafer*) and the monk-parakeet (*Myiopsitta monachus*) which are close species to those we have studied and that are also successful invasive species in different kinds of environments (Williams & Giddings 1984; Strubbe & Matthysen 2009). It might also be interesting to include very different species but that are also successful invaders in new environments, such as tilapias, fish that have been introduced in habitats varying in levels of dissolved oxygen or salinity (Firmat *et al.* 2012). This would enable assessing if rapid adaptation is more frequent in some taxa.

IV.2.2. Refining the phylogeographic studies

Including nuclear sequences to our phylogeographic study would probably bring more resolution to our current trees. This would enable us to identify more precisely the phylogenetic origin of introduced populations. Moreover, the study of nuclear sequences could allow the identification of possible introgressions between different genetic groups. For example, on Oahu we have found haplotypes related to two different clades. However, the

comparison of microsatellite loci showed that individuals on Oahu share similar alleles for these loci. Introgression might thus have occurred and the inclusion of nuclear sequences in our phylogenetic analysis could allow testing for this hypothesis. However, finding nuclear sequences variable enough to discriminate subspecies, remains a challenge in the case of our model species as their genomes have been little studied. Other introns or microsatellite flanking regions could be tested and pooled together to increase the number of variable sites if needed. In addition, increasing the sampling to cover more extensively the natural range of both species would allow defining more precisely the geographic limits of each phylogenetic group obtained.

IV.2.3. Studying other phenotypic traits and their heritability

As mentioned earlier, other phenotypic traits are likely to be involved in the successful establishment of a population into a novel environment. The comparison between introduced populations of other phenotypic traits than morphology might thus allow finding evidence of rapid adaptation where none could be find with the study of morphological traits. Examples of traits that could be selected in new environments include metabolic rates and seasonal changes in fat accumulation as they are associated with thermal regulation and can thus favor establishment in new climatic ranges (Thabethe *et al.* 2013). Changes in behaviors such as feeding behavior or anti-predator behavior are also expected to affect the success of establishment in new conditions (Yonekura *et al.* 2007; Anson & Dickman 2013). Finally, the adjustment of life history traits can also be involved in the adaptation to a novel environment (Bøhn *et al.* 2004; Amundsen *et al.* 2012).

The study of the heritability of each trait studied, either thanks to common garden experiments or animal models, would also be interesting to assess the role of phenotypic plasticity in the phenotypic differentiations that could be observed with the study of the traits mentioned above.

IV.2.4. Describing the different environments studied

During this thesis, we used climatic data to establish the degree of similarity or dissimilarity between the different environments we studied. Although the climate of a region can be a good indicator of the kind of vegetation present, it is not very precise and we have probably

missed a lot of important ecological factors acting on our study species. It is thus possible that the environments we considered as replicate are in fact different in some ecological aspects that are important for our study species. It would thus be interesting to collect data on the species (plants and animals) that compose the ecosystem found in the different environments we studied. Moreover, it would also be interesting to collect precise climatic data such as monthly temperatures and rainfall in each environment. This would allow really comparing the different environments and assessing their degree of similarity. In addition it would be possible to test whether some ecological factors drive the morphological patterns we observed.

IV.2.5. Using complementary approaches

Finally, complementary approaches could be used to check some hypotheses formulated during this thesis. We have seen earlier examples of classical methods used to assess if a phenotypic trait is adaptive in a specific environment and heritable. In our two case species, we will not be able to use animal models before some time as we do not have the data necessary yet and acquiring them would require the follow-up of some populations for several generations. Reciprocal transplants have also to be excluded. Indeed, the release of new individuals in an introduced population could favor the invasiveness of this population by increasing its genetic diversity (Sakai *et al.* 2001; Facon *et al.* 2008). Common garden experiments are however worth considering provided that our model species can be raised in captivity and that necessary authorizations would be given. As both species are sold as cage birds, raising them in captivity probably does not present too many technical difficulties. Concerning legal and ethical constraints, raising captive Ring-necked parakeets in Paris for example, would have small consequences in the case of an accidental release as they are already present in Europe. However, there might be some concerns about the raising of Red-whiskered bulbuls which might escape and become invasive. If common garden experiments were possible, they could be used to test if the morphological differences observed between the two sides of Mauritius are caused by adaptation. We could also test whether parakeets from Barcelona, Paris and Heidelberg are better adapted to the temperatures found in their introduced range than individuals from the other populations.

Besides these experimental approaches, the study of a large number of loci thanks to genome scans approaches is also worth considering. For example, restriction site associated sequencing (RADSeq) allows identifying thousands of genetic markers randomly distributed

across the genome in a group of individuals (Davey *et al.* 2010). The study of population genetics statistics such as FST can then be used to assess fluctuations in differentiation of populations across the whole genome. This allow identifying regions of the genome that have changed more rapidly than they would have been under genetic drift alone, *i.e.* parts of the genome that are under selection (*e.g.* Hohenlohe et al. 2010). Using RADSeq on our model species would therefore allow assessing if some populations have gone through rapid adaptation. If some portions are found to be under selection, it would however not be possible to know exactly which traits are evolving as our model species have not yet been entirely sequenced. However, genomes of other birds are available and they could be used to identify roughly the role of the regions of interest. RADSeq can also be used for phylogeographic analyses in species that have not been fully sequenced (*e.g.* Emerson et al. 2010). We could therefore use this technique to improve our phylogeographic study by the addition of new variable sites.

IV.2.6. Perspectives in the study of rapid adaptation in general

The study of rapid adaptation is only at its beginnings. We have showed that the comparative approach we used can allow identifying possible cases of rapid adaptation by first rejecting alternative hypotheses, which is easier that directly testing the hypothesis of adaptation. Moreover, new promising techniques such as RAD sequencing are getting available and will facilitate the discovery of cases of rapid adaptation. An interesting perspective would be to apply these approaches to the study of organisms for which rapid phenotypic changes have already been reported in order to assess if these changes have been caused by rapid adaptation. This will increase our knowledge on rapid adaptation as we might be able to estimate at which frequency it can occur and in which circumstances. Such knowledge will have implications both in the management of invasive species and in the conservation of endangered ones.

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Crédits photographiques :

- p 9 : Perruche à collier sous la neige – Stéphane Douady
- p 33 : Couple de bulbuls orphée – Clément Vulin
- p 45 : Festin dans un catalpa – Isabelle Muller
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Abstract

Recently, there has been a growing interest for rapid evolution and its potential role in ecological processes. For example, it has been hypothesized that rapid adaptation is a factor favoring the establishment of invasive species in new environments. If this hypothesis is true, it could have implications in the management and prevention of biological invasions. In vertebrates, lots of studies report cases of rapid phenotypic changes in invasive species following their introduction in a novel environment. However, because of the difficulties of directly testing for adaptation, very few of them were able to prove that these phenotypic changes result from rapid adaptation.

In this thesis, we were thus interested in assessing whether rapid adaptation can explain phenotypic changes observed in recently introduced populations. Instead of directly testing for adaptation, we tested for alternative hypotheses, which are easier to investigate. Indeed, a phenotypic difference observed between populations established in different environments can be caused by natural selection but also by phenotypic plasticity, by a different phylogenetic origin and by stochastic evolution (*i.e.* stochastic changes in allele frequencies in a population as the result of demographic processes such as founder effects and bottlenecks). Here, we studied two successful invasive bird species introduced in several kinds of environments. We described the morphology of individuals in these populations, and tested for the effects of historical factors (*i.e.* phylogenetic origin and recent demographic history) to explain morphological differences observed between populations.

In both species, our results show that stochastic evolution resulting of recent demographic history is likely to be the cause of the morphological differences observed. This was true for all the cases we studied except one. In this last case, neither a difference in phylogenetic origin, nor stochastic evolution could explain de phenotypic differences observed between two environments. It is therefore possible that rapid adaptation occurred in this case but the hypothesis of phenotypic plasticity remains to be tested.

In conclusion, with this work we highlighted that recent demographic processes can have an important role in causing morphological differentiation in invasive species. This role was probably underestimated in studies on rapid adaptation and should be taken into account in the future. We also showed that the comparative approach we used can allow identifying possible cases of rapid adaptation by first rejecting alternative hypotheses.